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Contents

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THE EFFECT OF VITAMIN A ON HEREDITARY HYPERKERATOSIS IN THE MOUSE[1]

BY F. CLARKE FRASER

Abstract

The formation of cysts in the sebaceous glands and follicle ends that occurs in "rhino" mice can be suppressed by massive vitamin A therapy. The significance of this finding is discussed in relation to the mode of action of the rhino gene.

Introduction

It was shown in previous communications (12, 2) that the simple recessive "rhino" (*hr*^{rh}) gene in the mouse causes a follicular hyperkeratosis associated with depilation and subsequent cyst formation in the hair follicles and sebaceous glands. Transplantation experiments showed that rhino skin when grafted at birth to a normal host would behave autonomously in the center of the graft, but that around the edges of the graft, where rhino skin grew in close proximity to normal skin, the genetically rhino follicles behaved like normal follicles and did not lose their hair or undergo cyst formation. This suggested that there is a cell-diffusible substance produced by normal skin cells but not present in the blood stream that is necessary for the normal maintenance of the cutaneous epithelium. Skin cells homozygous for the rhino gene presumably cannot produce this substance in quantities sufficient to keep the skin normal, although apparently they can utilize it when it is supplied by adjacent normal skin cells.

Because of the histological similarities between rhino skin and that of animals fed diets deficient in vitamin A (3, 7, 4, 9, 13), it was thought that the substance postulated to be deficient in rhino skin might be a metabolite of vitamin A. If this is so, then increasing the blood concentration of vitamin A in rhino mice might lead to an increased production of the substance and cause a regression of the rhino condition towards normal.

A similar problem exists clinically, with regard to the treatment of several types of hereditary hyperkeratoses with vitamin A (10, 11, 5). The high degree of variability between patients in blood levels of vitamin A, dark adaptation, and response to treatment with vitamin A in cases of pityriasis rubra and Darier's disease (two hereditary diseases involving a cutaneous hyperkeratosis) may be accounted for on the assumption that in different

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Contribution from the Department of Genetics, McGill University, Montreal, Que.

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cases different genes may be involved, each interfering with the vitamin A metabolism at a different point. The skin may manifest a lack of vitamin A because it is deficient in the blood (due to impaired absorption or liver function) or because it cannot be utilized by the skin cell. In view of the experiments about to be reported, it is thought that the rhino mouse may serve as an experimental medium for investigation of the latter possibility.

Experiments previously reported (2) in which rhino mice were fed massive doses of vitamin A in the form of ling liver oil gave results that were rendered inconclusive by the systemic toxicity of the treatment. The question of whether this toxicity was associated with the vitamin A or was entirely due to some other constituent in the preparation has been answered by the demonstration of Moore and Wang (8) that crystalline vitamin A is itself toxic.

Experimental Results

(a) *Systemic Toxicity*

The mice in this experiment were fed a highly purified distillate of the natural esters of vitamin A containing 500,000 International Units of vitamin A per gram, suitably diluted with Mazola oil and administered orally every other day through a graduated medicine dropper. Control litter mates were fed equivalent amounts of Mazola oil in a similar way.

Throughout the experiments the dosage was maintained at a maximum compatible with the general health of the mouse as estimated by the absence of any of the outward signs of systemic toxicity discussed below. Doses of the order of 5000 I.U. every other day for about one month to adult rhino mice caused the skin to become hyperemic, oily, and covered with fine powdery scales. The mice at this time became restless, easily excited, and scratched themselves a good deal as if there was some cutaneous irritation. If treatment was continued there was a rapid loss of weight and the animals became listless, emaciated, and eventually moribund within two weeks of the appearance of toxic signs. The only consistent macroscopically noticeable post-mortem findings other than the cutaneous effects described below were a marked enlargement of the spleen and a less pronounced enlargement of the liver. The degree of enlargement of these organs varied with the dosage and the length of time the animals survived treatment. Associated histological changes are described in Section *b* (1). Discrepancies between these findings and those described in other reports of systemic vitamin A toxicity (14, 6, 8) may be due to the more chronic nature of the treatment in the present case. This aspect of the problem will be investigated more fully.

The dosage level that will permit administration over long periods without external signs of systemic toxicity has been established as being of the order of 500 to 1000 I.U. every other day. Although enlargement of the liver and spleen does occur at this dosage level, several mice have survived for as long as eight months at this dosage without showing any outward signs of the treatment except the skin changes about to be described.

(b) *Skin Effects*

(1) *Feeding Vitamin A*

Experimental results indicate that a high blood level of vitamin A is associated with inhibition of the growth of the sebaceous and follicle-end cysts that ordinarily develop in rhino skin following hair loss. The records of one pair of mice will be presented in some detail to demonstrate this characteristic response to treatment. A rhino male, 2804, was fed vitamin A concentrate beginning at an age of 17 days with a dose of 1250 I.U. every other day, increased to 5000 I.U. every other day at five weeks. Male 2807, a rhino litter-mate of this mouse was used as a control. At eight weeks of age the skin of the treated animal was noticeably pinker, more oily, thinner, and smoother than that of the control, which had begun to develop the characteristic rhino thickening and wrinkling (Figs. 1 and 2). In another week the skin of the treated mouse was very pink, and, in the dorsal shoulder region, covered with fine powdery white flakes. The mouse was breathing rapidly and the liver, which could be seen through the skin, was noticeably enlarged. A similarly treated litter-mate, after a rapid loss of weight, died at this time. Consequently the dose was reduced to 1250 I.U. every other day until the age of 21 weeks, when the skin of the treated animal was still soft, thin and smooth, although that of the control continued to get thicker and more wrinkled. Here the dose of vitamin A was reduced to about 600 I.U. every other day and continued at this level until death of the animal at 50 weeks. At death the spleen of the treated animal was tremendously enlarged, weighing 0.7 gm. as compared with a weight of 0.1 gm. for the spleen of the control. The organ appeared congested with blood but was very firm and the cut surface showed many dense white nodules. The liver was also enlarged but not as much as the spleen. Its surface was speckled with white spots and there were several hemorrhagic areas visible.

I am indebted to Dr. W. E. Finkelstein of the Department of Pathology, McGill University, for the following histopathological observations on these organs, and hereby extend my thanks to him for his generous co-operation in this respect. "One section of liver from male 2804 is examined. Sections are stained with Masson's trichrome, Masson's hemalum, phloxine and saffron, Weigert's elastic tissue and Wilder's reticulum stain. There is severe alteration, distortion and loss of the normal hepatic architecture. Broad interlacing bundles of moderately stout collagen fibres forming a loose network intersect throughout the section, enclosing larger and smaller irregularly outlined areas of surviving parenchyma. The spaces in this collagenous network are heavily infiltrated with large numbers of both acute and chronic inflammatory cells. Polymorphonuclear leukocytes and large mononuclear cells are present in approximately equal numbers. These are large, round to oval with prominent, round, oval or reniform nuclei. One of the larger portal veins shows marked cellular infiltration of its wall by polymorphonuclear cells and macrophages. The bands of fibrosis include the portal tract and apparently also incorporate the central veins, although it is difficult to distinguish with certainty between

portal and central veins. There is minimal proliferation of bile ducts in the portal tracts and they are largely obscured by the intensity of the surrounding inflammatory reaction. In several instances, the lining cells of the bile ducts show small brightly acidophilic droplets in their cytoplasm. At the borders of the bands of fibrous tissue collagenous fibres insinuate themselves for short distances between liver cells and in some places, the cells so enclosed are compressed, shrunken and in many instances necrotic. Similar hepatic cells are seen to be sequestered in the central portions of some of the zones of fibrosis. The surviving hepatic parenchyma shows loss of the usual radial pattern and the cells exhibit mild degenerative changes. Focal areas of necrosis are present throughout the surviving parenchyma often bordering on the bands of fibrous tissue but occasionally seen remote from them. In such areas, the liver cells are compressed and atrophic with strongly acidophilic cytoplasm; many show karyorrhexis and karyolysis. Occasional mitonecroses are noted. In some of these areas, the liver cells have completely disappeared, only a collapsed reticulin network remaining. A few of the liver cells show fine cytoplasmic vacuolation but this change is rare and not conspicuous. Sections of liver stained for bacteria by Glynn's method and with Ziehl-Nielsen carbol-fuchsin for acid-fast organisms are negative.

"In summary, the section examined shows severe degenerative, inflammatory and productive changes. The histological pattern of bands of fibrous tissue enclosing larger and smaller masses of persistent hepatic parenchyme is indistinguishable from that seen in active cirrhosis.

"One section of spleen from male 2804, stained with Masson's hemalum, phloxine and saffron is examined. The spleen is hyperemic and the sinusoids are filled with red cells. Lymphoid follicles are small and reduced in number. The red pulp is highly cellular being packed with numerous lymphoid and reticular elements. Many of the cells are lymphocytes while numerous large mononuclear cells are present. Occasional mitotic figures are present, polymorphonuclear leukocytes are present in moderate numbers and megakaryocytes are also present. The alteration in the spleen is non-specific in character and may be characterized as hyperplasia of the pulp."

The lungs were congested and showed many petechial hemorrhages. No pathological lesions were found in the control animal.

Biopsy specimens taken at 31 weeks showed that in the skin of the treated mouse there was an almost complete absence of cysts in the sebaceous glands and follicle ends, although there were many hair canal cysts present. Sections of skin removed at death (50 weeks) from the treated animal showed that the only change that had occurred since 31 weeks was that the hair canal cysts

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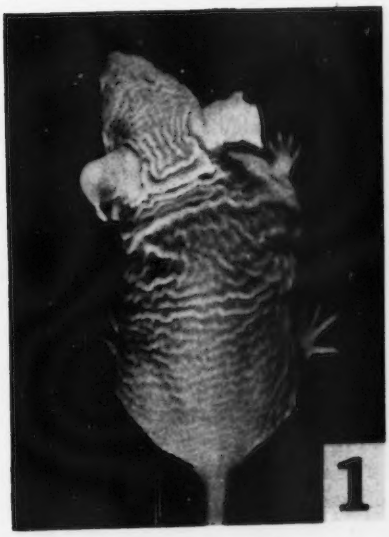
FIG. 1. *Male 2807, untreated rhino, eight weeks old.*
FIG. 2. *Male 2804, rhino treated with vitamin A, same age.*
FIG. 3. *Skin from rhino male 2807, 50 weeks old.* ×85.
FIG. 4. *Skin from rhino male 2804, same age, treated with vitamin A.* ×85.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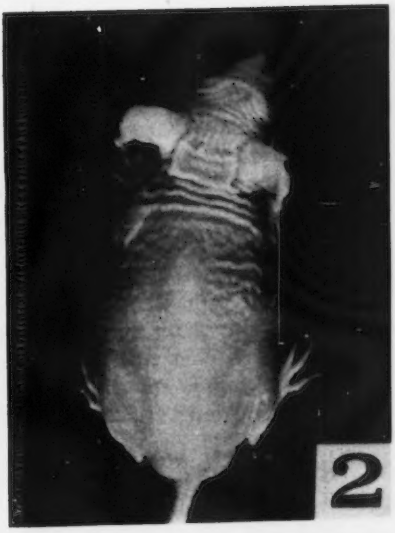
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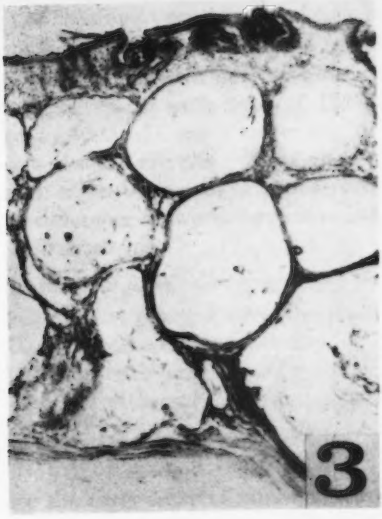
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had become smaller – as they had also in the control – and the connective tissue of the corium had become a little denser (Figs. 3 and 4). In the control animal, on the other hand, the sebaceous and follicle-end cysts had become much larger during this interval.

The vitamin A treatment does not seem to cause much, if any, actual regression of the sebaceous and follicle-end cysts, it merely stops them from developing any further. This conclusion was drawn from two animals in which treatment was started at 10 weeks of age, well after cyst formation had begun. Biopsy specimens taken at the beginning of treatment and after 19 weeks of treatment showed that the degree of cyst formation was about the same in the treated animal before and after treatment, but had progressed noticeably in the control animals over the same period.

This inhibitory effect of the treatment on cyst formation is a regular phenomenon, which has been observed, at various stages, in a total of 15 rhino mice, each one compared with an untreated rhino litter-mate. No such inhibition has ever been seen in the absence of treatment in any of the hundreds of rhino animals observed by the author, and it seems clearly established that the effect is a consequence of treatment.

So far no animal in which treatment was started before the time of hair loss has survived. In four rhino mice that were fed 300 I.U. of vitamin A every other day starting at three days of age and that died on the 30th day, no effect of treatment on the rate of hair loss was noted.

Two nonrhino animals from the rhino strain (which is maintained by breeding rhino males with heterozygous sisters) were fed vitamin A starting at four weeks of age with doses of 1250 I.U. every other day. This was increased to 4000 I.U. every other day at six months, and maintained here till the age of seven months. Throughout this period no effect on the outward appearance of the hair coat was observed, nor did histological examination show any difference between the skins of the treated mice and those of control nonrhino litter-mates.

(2) *Feeding Carotene*

No significant changes were observed in the skins of two rhino mice fed 2500 I.U. of carotene every other day for 10 weeks beginning at five weeks of age. Nor were there any signs of systemic toxicity noticed during treatment or at autopsy. Control litter-mates were fed corresponding amounts of Mazola oil.

(3) *Feeding Vitamins A and C*

Since the early signs of vitamin A intoxication resemble those occurring in scurvy (8, 14), the effect of feeding vitamin C to mice being treated with vitamin A was observed. Two rhino members of a litter were fed 1250 I.U. of vitamin A every other day beginning at the age of four weeks. Two other rhino animals from the same litter were fed similar doses of vitamin A, with 1 mgm. of vitamin C in aqueous solution being given concurrently. No effect of the vitamin C on either the cutaneous signs of vitamin A treatment or the

enlargement of liver and spleen could be detected after two months of treatment. This result is in agreement with those of Moore and Wang (8) who performed similar experiments in rats.

Discussion

Although the above experiments were largely of an exploratory nature, they have clearly demonstrated that a high blood level of vitamin A is associated with suppression of the process leading to cyst formation in the sebaceous glands and follicle ends in the rhino mouse. Whether this effect is due to the fact that the treatment supplies the rhino skin with some metabolite of vitamin A that is necessary for maintenance of the skin in a normal condition is not entirely proved. The possibility that the retardation in cyst formation is a secondary manifestation of the systemic toxicity has not been excluded. However, the fact that a similar dosage of vitamin A in nonrhino mice does not seem to retard hair growth argues against this interpretation. The question of why the treatment affects cyst formation in the sebaceous glands and follicle ends and not in the hair canal also remains unanswered. Perhaps the dosage of vitamin A necessary to affect the formation of hair canal cysts is above the level of toxicity, and kills the mouse before it is able to cure the condition.

In conclusion, it can be stated that the experimental results reported above are compatible with the assumption that the presence of the rhino gene in the homozygous condition is associated with a decreased ability of the skin cells to utilize some metabolite of vitamin A necessary for their normal maintenance. Enzymatic studies on the utilization of vitamin A by rhino and nonrhino skin respectively should provide further relevant information. If the gene does act by causing a metabolic deficiency analogous to those studied so profitably in the lower organisms (1), it might provide a useful tool for the analysis of the intracellular metabolism of vitamin A.

Acknowledgments

I would like to acknowledge my indebtedness and gratitude to the National Research Council of Canada, which provided financial assistance, and to Dr. M. Demerec and the Long Island Biological Association for financial aid and the use of the laboratory facilities at Cold Spring Harbor during part of this work.

I would also like to express my sincere thanks to Dr. K. D. Hickman of Distillation Products Inc., who kindly supplied the vitamin A concentrate and the firm of Ayerst, McKenna and Harrison who provided the carotene used in this study.

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SOME EFFECTS OF P^{32} ON THE DEVELOPMENT OF *DROSOPHILA*[1]

By T. J. Arnason,[2] R. L. Irwin,[3] and J. W. T. Spinks[4]

Abstract

When freshly laid *Drosophila* eggs were placed in a graded series of P^{32} concentrations in the culture media, death of treated individuals occurred most frequently at the end of the larval period and in the pupal stages. No adults emerged from cultures having an initial concentration of 0.65 rutherford (rd.) or higher per ml. food. The calculated concentration of P^{32} expected to reduce emergence of adults to 50% is 0.120 rd. per ml. A dosage of 1.30 rd. per ml. prevents transformation of larvae to pupae. Three out of 132 X-chromosomes from males reared in medium having an initial concentration of 0.0325 rd. per ml. carried new recessive lethal "genes".

Introduction

In planning experiments designed to reveal the mutagenic efficiency of radioisotopes, it is desirable to know the tolerance limit of the organism for the isotopes since maximal mutation frequency may be expected at the highest applied dosages. *Drosophila melanogaster* appears to be a particularly suitable organism for testing since exact determinations of the frequency of X-ray induced mutations have been made repeatedly (2). It is thought, therefore, that the establishment of the minimal dose of radiophosphorus that is lethal to *Drosophila* will be of some interest.

Materials and Methods

Eggs of wild type (Canton Special Stock) were collected by means of a 5 ml. shell vial apparatus, illustrated in Fig. 1. Twelve to fourteen females and

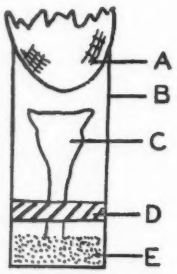

Fig. 1. *Vial for collection of eggs. A, cotton and gauze plug; B, shell vial, 5 ml.; C, filter paper wick; D, cork slice; E, banana–yeast mixture.*

[1] *Manuscript received in original form December 2, 1948, and, as revised, April 8, 1949. Contribution from the Departments of Biology and Chemistry, University of Saskatchewan, Saskatoon, Sask.*

[2] *Associate Professor of Biology.*

[3] *Research Assistant.*

[4] *Professor of Physical Chemistry.*

several males were placed in the vial where they were able to feed on the juice of a banana and yeast mixture, drawn by capillarity from the lower part of the vial. Flies were kept for 24 to 48 hr. in the vials prior to egg collection. After this period of acclimatization the flies appeared to be stimulated in egg production.

The eggs were laid on the cork, which had been lightly smeared with banana pulp. The eggs were removed by means of fine steel needles and transferred to the surface of the medium, containing radiophosphorus, in a 5 ml. shell vial. All eggs used were collected within eight hours of laying.

The culture medium employed was the usual (7) corn meal, agar, molasses, and yeast mixture. Radiophosphorus, in the form of phosphoric acid was mixed with the medium, the total volume of medium plus P^{32} being one ml. per vial. Nine series of vials, labelled I to IX, were used, each series consisting of five vials containing concentrations of P^{32} as listed in Table I. Fifteen eggs were placed in each vial. The vials were placed in an incubator kept at 23° ± 2° C. and a high humidity (to prevent drying of the cultures).

TABLE I

CONCENTRATIONS OF P^{32} USED, RD. PER VIAL*

Vial	A	B	C	D	E
P^{32} activity, rd.	0	0.065	0.325	0.65	1.3

* *We are grateful to W. D. Murray for radioactivity measurements.*

All the larvae from Series I to VI were extracted after 2.5, 5, 7, 9, 10, and 12 day periods respectively. Series VII to IX were allowed to reach the adult stage and the adults were used for emergence and breeding tests.

The extracted larvae from Series I to VI were washed twice with water and once with 70% alcohol after which they were placed on a cover slip and warmed over a lamp with a drop of 3 : 1 alcohol – acetic acid mixture. This latter process killed, expanded, and fixed the larva. An ocular micrometer in a binocular microscope was used in measuring larval lengths.

The amount of P^{32} in a specimen was measured by placing the cover slip, on which the larva was fixed, in a reproducible position under the window of an end-on Geiger-Müller chamber (mica window, 3 mgm. per sq. cm.) and counting it. The chamber was connected to a scale of 64 scaling circuit and any sample was counted for a sufficient length of time to give approximately 10,000 counts (standard deviation is then about 1%). By placing the cover slip at a suitable distance below the counter window, the rate of counting was kept below 2000 counts per minute and the correction for resolving time of the tube (2×10^{-4} sec.) was less than 1%.

The natural rate of the counting tube (so-called "background") was allowed for by subtracting it from the total counting rate for any sample. The

radioactive decay of P^{32} (half life 14.3 days) was allowed for by taking an aliquot of the original stock solution, evaporating it to dryness, and counting it immediately before and after counting the unknown material. The aliquot of the original thus serves as a standard and eliminates the necessity of correcting for the decay of the P^{32} or for small fluctuations in the counter. In some experiments, counter fluctuations were allowed for by comparing the unknown with a uranium oxide standard. Absolute rates of disintegration were obtained by multiplying the observed counting rate by a geometry factor obtained by calibrating the counter with an RaD + E standard from the Bureau of Standards (8).

The weight of the larvae (< 1.2 mgm.) is such that self absorption corrections are negligible (self absorption half thickness for P^{32} radiations is approximately 300 mgm. per sq. cm.) and it is thus not necessary to ash the sample before counting.

As flies from Series VII to IX emerged, both sex and time of emergence were recorded and individuals to be used for breeding were transferred immediately. Some adults were dissected to test for radioactivity in various parts of the body. Twenty-six days after the treatment began it was judged that no further emergence could be expected and all remaining pupae and puparia were extracted. Their radioactivity was measured and the amount of pupal differentiation noted. An arbitrary measurement of the amount of development was used.

Stage 0 : larval development only.
Stage 1 : puparia formation but no development of gross features.
Stage 2 : differentiation of gross features (head, thorax, abdomen).
Stage 3 : development of gross features but incomplete differentiation of minute structures.
Stage 4 : full development, no emergence.
Stage 5 : full development and emergence.

Results

1. *Measurement of Radioactivity and Development in Larvae*

The measurements of larval length and total contained activity are recorded graphically in Figs. 2 to 5. It appears that, for all initial concentrations, the larger the larva, the greater the amount of radioactivity contained.

The variation of average larval length with time and initial radioactivity is recorded in Table II. From the results it is apparent that the P^{32} of these concentrations had little noticeable effect on the growth of the surviving larvae.

2. *Measurement of Radioactivity and Development in Pupae*

The amount of radioactivity in pupae that had failed to emerge by the 26th day from the start of treatment is recorded in Table III. In the B concentration (not recorded in the table), only three pupae had failed to emerge.

The amount of P^{32} in pupae is roughly proportional to the concentration in the medium. Differentiation of pupae is decreased as the concentration of radioactivity rises.

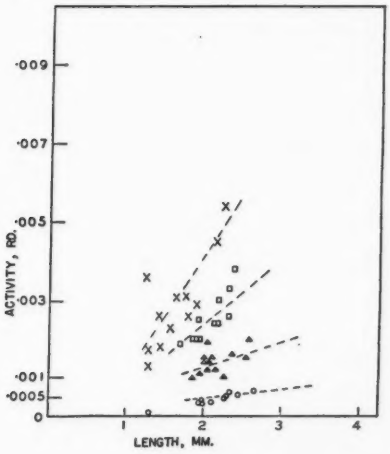

FIG. 2.

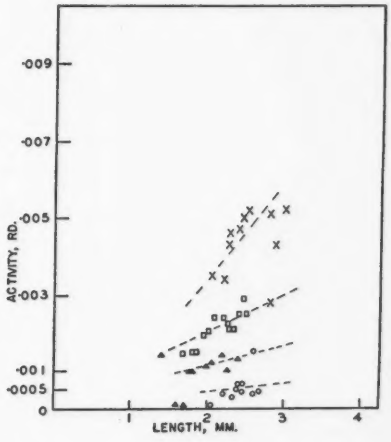

FIG. 3.

FIG. 2. *Larval length at five days, plotted against contained P^{32} activity in rd. Original concentration P^{32} in medium in rd. per ml.* ○, *0.065;* △, *0.325;* □, *0.65;* ×, *1.3.*

FIG. 3. *Larval length at seven days, plotted against contained P^{32} activity in rd. Original concentration P^{32} in medium in rd. per ml.* ○, *0.065;* △, *0.325;* □, *0.65;* ×, *1.3.*

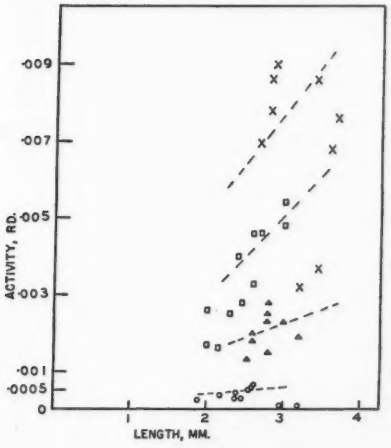

FIG. 4.

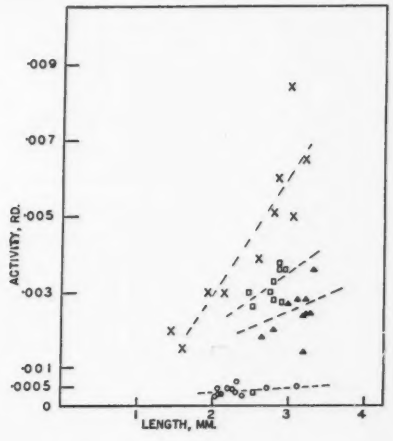

FIG. 5.

FIG. 4. *Larval length at nine days, plotted against contained P^{32} activity in rd. Original concentration P^{32} in medium in rd. per ml.* ○, *0.065;* △, *0.325;* □, *0.65;* ×, *1.3.*

FIG. 5. *Larval length at 10 days, plotted against contained P^{32} activity in rd. Original concentration P^{32} in medium in rd. per ml.* ○, *0.065;* △, *0.325;* □, *0.65;* ×, *1.3.*

TABLE II

P^{32} EFFECTS ON GROWTH OF LARVAE

Initial activity P^{32}, rd./ml.	Average larval length, mm. The number of larvae extracted from each vial, in brackets									
	2.5 days		5 days		7 days		10 days		12 days	
0	1.83	(15)	2.27	(12)	2.46	(13)	2.81	(12)	3.02	(11)
0.065	1.21	(14)	2.01	(12)	2.51	(10)	2.47	(13)	2.31	(13)
0.325	1.44	(13)	2.15	(13)	1.92	(13)	2.87	(13)	3.09	(11)
0.65	1.20	(12)	1.87	(12)	2.12	(14)	2.51	(13)	2.74	(10)
1.3	1.18	(13)	1.67	(13)	2.47	(10)	2.69	(8)	2.46	(10)

TABLE III

DEGREE OF DEVELOPMENT AND AMOUNT OF RADIOACTIVITY IN INDIVIDUAL PUPAE (AT 26 DAYS)

Concentration C (0.325 rd. per ml.)		Concentration D (0.65 rd. per ml.)		Concentration E (1.3 rd. per ml.)	
Development of pupa, stage	Total radioactivity in pupa and puparium, rd.	Development of pupa, stage	Total radioactivity in pupa and puparium, rd.	Development of pupa, stage	Total radioactivity in pupa and puparium, rd.
4	0.00023	1	0.00080	0	0.00090
4	0.00047	1	0.00077	0	0.00170
4	0.00036	1	0.00106	0	0.00530
3	0.00038	2	0.00046	0	0.00320
3	0.00033	1	0.00098	0	0.00540
				0	0.00100
				1	0.00098
Average	0.00035	—	0.00081	—	0.00264

3. *Measurement of Radioactivity in Whole and Dissected Adults*

Activity measurements were made on 10 flies reared on medium of B concentration. Three of the flies emerged from pupae that had been transferred to a medium containing no activity. Recorded activities in these three were as high as in flies that emerged in vials containing P^{32}.

The average percentages of the total radioactivity appearing in the main divisions of the body were as follows: head, 16%; thorax including appendages, 47%; abdomen, 37%. Less than 5% of the activity was in the wings and legs. The results are recorded in Table IV.

4. *Pupation*

All the individuals developing in Series VII to IX were grouped together for observations on pupal differentiation and emergence of adults. The numbers of pupae developing in the various concentrations of radioactivity are recorded in Table V. At the highest activity very few larvae managed to pupate. The percentage of pupae formed as a percentage of the eggs seeded rises as activity concentration declines.

TABLE IV

P^{32} ACTIVITY, IN RD., IN YOUNG ADULT *Drosophila* REARED ON MEDIUM CONTAINING 0.065 RD. PER ML.

Fly number	Head	Thorax	Wings	Legs	Abdomen	Total	Fly number	Whole fly
1	0.000057	0.000148	0.000000	0.000009	0.000126	0.000340	6	0.000288
2	0.000060	0.000107	0.000007	0.000012	0.000176	0.000362	7	0.000325
3	0.000052	0.000172	0.000013	0.000005	0.000128	0.000370	8*	0.000324
4	0.000051	0.000140	0.000002	0.000022	0.000114	0.000329	9*	0.000278
5	0.000055	0.000177	0.000001	0.000013	0.000097	0.000343	10*	0.000423
Average	0.000055	0.000149	0.000005	0.000012	0.000128	0.000349	—	0.000328

* *Transferred to inactive medium before emergence of adults.*

TABLE V

NUMBERS OF PUPAE FORMED FOR VARIOUS INITIAL ACTIVITIES OF RADIOPHOSPHORUS

Initial activity, rd.	0	0.065	0.325	0.65	1.3
Number of eggs seeded	45	45	45	45	45
Number of pupae formed	38	31	27	17	3
Percentage of pupae formed as percentage of eggs seeded	84	69	60	38	7

5. *Inhibition of Pupal Development in High P^{32} Concentration*

The number and the average degree of development of the pupae remaining in the 26-day old cultures of Series VII to IX is recorded in Table VI. By this time adults had already emerged in the A, B, and C concentrations as is reported in Table VII. Very few pupae were formed in Concentration E, and little pupal differentiation occurred in D.

TABLE VI

PUPAL DEVELOPMENT FOR DIFFERENT INITIAL ACTIVITIES OF P^{32}

Initial activity P^{32}, rd. per ml.	0	0.065	0.0325	0.65	1.3
No. of pupae remaining in 26-day old culture	1	3	24	17	3
Average stage of development of pupae remaining	4	3–4	2–3	1	0–1

6. *Emergence*

Results of emergence for Groups A, B, C, D, E in Series VII to IX are listed in Table VII. At concentrations of 0.65 rd. of P^{32} or higher, no adults emerged; at the 0.325 rd. concentration 6.6% emergence was recorded but

all the flies died within three days. However, at 0.065 rd., the next lower concentration, 62.2% of the treated individuals emerged.

TABLE VII

NUMBER OF FLIES EMERGING FROM DIFFERING INITIAL ACTIVITIES OF P^{32}

Initial activity of P^{32}, rd. per ml.	Number of flies emerging														
	0			0.065			0.325			0.65			1.3		
Time of emergence, days	♂	♀	Total	♂	♀	Total	♂	♀	Total	♂	♀	Total	♂	♀	Total
15	7	12	19	2	4	6	1*	0	1	0	0	0	0	0	0
17	10	5	34	1	10	17	0	0	1	0	0	0	0	0	0
19	0	2	36	0	2	19	2*	0	3	0	0	0	0	0	0
20	0	1	37	2	5	26	0	0	3	0	0	0	0	0	0
22	0	0	37	0	2	28	0	0	3	0	0	0	0	0	0
26	0	0	37	0	0	28	0	0	3	0	0	0	0	0	0
No. of eggs	45			45			45			45			45		
% Emergence	82.2			62.2			6.6			0			0		

* *Died three days after emergence.*

7. *Lethal Mutations**

Wild-type larvae of the Canton Special stock, which has a low spontaneous mutation rate, were reared in culture medium containing 0.0325 rd. P^{32} per ml. Forty-four treated males were successfully mated to C l B females. Three of 132 C l B daughters from these crosses gave aberrant sex ratio as follows:

95 ♀♀ : 0 ♂; 51 ♀♀ : 0 ♂; 28 ♀♀ : 1 ♂.

It is concluded that two of 132 tested X-chromosomes had new recessive lethals; in a third X-chromosome, a semilethal mutation may have occurred. The observed proportion of male gametes carrying new recessive lethal mutations is 0.015 or 0.023, depending on whether the marginal lethal is counted or not. The mutation rate for sex-linked recessive lethals from sperms irradiated with 1000 *r* of X rays is 0.0289 (2).

Discussion

Radiophosphorus supplied to *Drosophila* in the food medium for the whole of the developmental period obviously does not have an immediate lethal effect on larvae at any of the concentrations used. Above a certain level, the cumulative effect of continued radiation kills old larvae, pupae, and adults. Our estimates of lethal effects are based on determinations of the initial concentration of P^{32} in the food medium required to kill individuals prior to emergence of adults. On this basis a dosage of 0.65 rd. per ml. is completely lethal and the calculated mid-lethal dose is 0.120 rd. per ml. (Fig. 6).

* *We are indebted to Hulda Regehr for these observations.*

Most of the deaths occurred in late larval and in pupal stages. This does not necessarily imply greater sensitivity at these stages, since dosage effects are cumulative and these (older) individuals have absorbed more r.e.p.* than

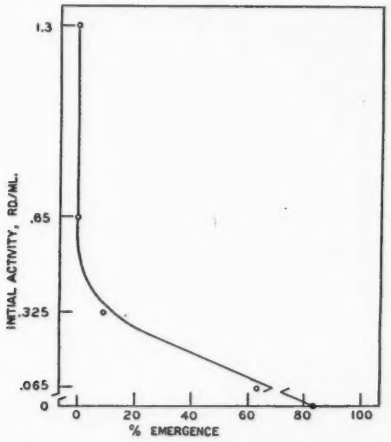

FIG. 6. *Percentage of emergence versus initial activity in rd. per ml.*

have the (younger) larvae. Mavor (3) found that a much smaller dosage of X rays was required to kill eggs, larvae, and young pupae than was needed for older individuals.

The studies on pupal differentiation indicate that the amount of pupal development is roughly in inverse ratio to the P^{32} dosage. The failure to differentiate may result from effects on earlier larval stages as well as from direct effects of radiations on the pupae. When pupation fails to occur one of the possible mechanisms is by radiation effects on the ring gland whose hormone is known to influence pupation (1, 5).

It is probable that radiation effects include disruption of enzyme systems and inhibition of various syntheses necessary for pupal differentiation. Altered cell division rates may also be important, particularly in the pupal stages. Normally, according to Poulson (6), few divisions occur in larvae more than 12 hr. old, and larvae younger than that have received relatively little radiation even at the highest dosage used.

Acknowledgments

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* *Roentgen-equivalents-physical (4).*

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THE PROVENTRICULAR REGION OF *TETRAOPES TETRAOPHTHALMUS* FORST. (COLEOPTERA: CERAMBYCIDAE)[1]

By W. W. Judd[2]

Abstract

The proventricular region of *Tetraopes tetraophthalmus* Forst. is relatively poorly developed, consisting merely of the posterior end of the stomodaeum, which tapers toward the mid-gut and is invaginated into the anterior end of the mid-gut to form the oesophageal valve. It is of the type found in inactive insects that feed on plants and in which the crop is small.

Materials and Methods

Adult specimens of the milkweed beetle, *Tetraopes tetraophthalmus* Forst., were collected from the leaves and stems of the common milkweed, *Asclepias syriaca* L., growing along roadsides and in fields adjacent to the campus of McMaster University, Hamilton, Ont., Aug. 1 and 2, 1947. They were identified with a key to the genus *Tetraopes* in Blatchley (2). When brought into the laboratory, the beetles were placed in a cyanide jar. As soon as active movement had ceased each beetle was removed from the jar, the legs and wings were clipped, and the dorsal integument was cut, with a pair of fine scissors, along the mid line of the thorax and abdomen. Half the beetles were placed in 70% alcohol. The others were put in Bouin's fixative for about 12 hr. after which they were washed in repeated changes of 70% alcohol and then stored in 70% alcohol.

The gross structure of the stomodaeum and its relation to the mid-gut were studied in specimens preserved in alcohol. A beetle from the alcohol was pinned to a layer of wax in a watch glass and covered with water. The sides of the thorax and abdomen were pinned outward to disclose the digestive tract and it was found necessary to cut the head longitudinally and pin its halves outward in order to disclose the anterior portion of the crop.

Mounts of the lining of the stomodaeum were made from specimens preserved in alcohol. The digestive tract of a beetle was removed from the body cavity and the mid-gut was gently removed from its attachment to the oesophageal valve. One point of a fine pair of scissors was inserted into the valve and the stomodaeum was cut longitudinally along the length of its dorsal wall. The stomodaeum was then placed in a strong solution of potassium hydroxide for several hours until the muscles and other soft tissues had been dissolved. The hydroxide was washed out with water and the remaining sclerotized intima of the stomodaeum was washed with a series of alcohols of increasing strength. The alcohol was washed out with xylol and the intima was mounted in Canada balsam.

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Serial transverse sections of the stomodaeum were made from beetles that had been fixed in Bouin's fixative. They were sectioned at 10μ and were stained with haematoxylin and eosin.

Description

The stomodaeum of the beetle consists of a short, narrow oesophagus and an oval crop. The oesophagus (Fig. 2 – O) leads from the pharynx and is about 0.2 mm. wide and at its posterior end widens into the oval crop (C), which is about 2 mm. long. Most of the crop is enclosed within the head of the insect and only about one-quarter of its length extends through the foramen magnum of the head (Fig. 4 – C, F) into the thorax. The posterior end of the crop lies embedded in the anterior end of the mid-gut (Fig. 2 – MG). At this point the stomodaeum is sharply constricted and projects into the lumen of the mid-gut in the form of a rosettelike oesophageal valve with a narrow opening (Fig. 4 – V). The proventricular region of the stomodaeum is not distinguishable from the rest of the crop externally and consists merely of that portion at the posterior end of the crop that narrows toward the oesophageal valve.

The lining of the stomodaeum consists of a sclerotized intima (Fig. 1). In the crop it bears several low longitudinal ridges (LR). As these approach the constriction of the oesophageal valve (V) they anastomose to produce ridges that are fewer in number and finally, at the valve, they end in six poorly defined flaps (FL) that project into the mid-gut. The posterior half of the intima of the stomodaeum, including the longitudinal ridges and the oesophageal valve, is beset with a coating of small flattened spines (Fig. 3 – S). They are arranged in a regular, overlapping pattern, each spine being about 5μ long, broad at the base and tapering abruptly to a point that projects posteriorly in the stomodaeum.

In transverse section the longitudinal ridges of the anterior part of the crop are seen to project into the lumen (Fig. 5 – LR). They are covered with a layer of sclerotized intima (I), which is irregular in outline and about 10μ thick. Within the longitudinal ridges is a single layer of irregular epidermal

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FIG. 1. *Sclerotized intima of posterior part of stomodaeum.*

FIG. 2. *Stomodaeum and anterior end of mid-gut.*

FIG. 3. *Spines of posterior part of stomodaeum.*

FIG. 4. *Posterior view of head showing stomodaeum (with mid-gut removed) projecting through foramen magnum.*

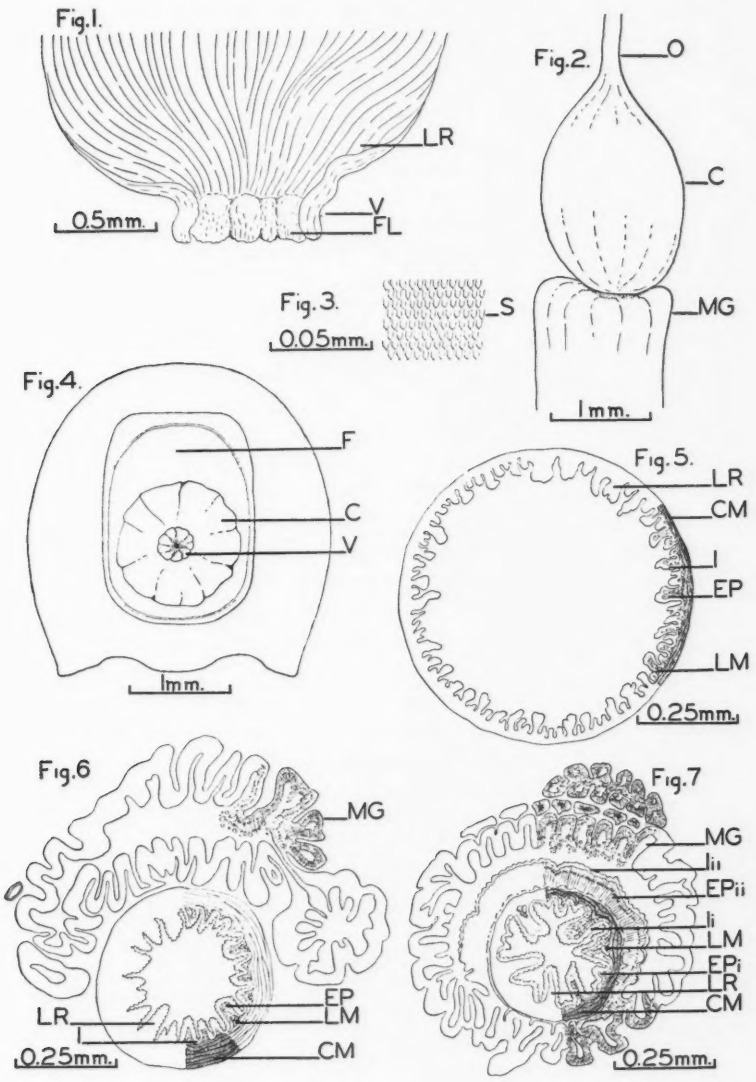
FIG. 5. *Transverse section of anterior portion of stomodaeum.*

FIG. 6. *Transverse section of posterior portion of stomodaeum.*

FIG. 7. *Transverse section of oesophageal valve and anterior end of mid-gut.*

C – crop; CM – circular muscle; EP – epidermis; EPi – inner epidermis of oesophageal valve; EPii – outer epidermis of oesophageal valve; F – foramen magnum of head; FL – flap of oesophageal valve; I – intima of stomodaeum; Ii – inner intima of oesophageal valve; Iii – outer intima of oesophageal valve; LM – longitudinal muscle; LR – longitudinal ridge; MG – mid-gut; O – oesophagus; S – spine of stomodaeum; V – oesophageal valve.

cells (EP). In some of the larger ridges are a few strands of longitudinal muscle (LM). The outer covering of the crop is a layer of striated circular muscle (CM) two or three strands thick. The posterior end of the crop is partly surrounded by a crescentic portion of the anterior end of the mid-gut (Fig. 6 – MG). The longitudinal ridges (LR) are fewer in number and larger than in the anterior part of the crop. The intima (I) presents a serrated



appearance owing to the presence of the small spines on its surface. The circular muscle (CM) is about twice as thick as in the anterior part of the crop and consists of four to six strands. The oesophageal valve lies within the anterior end of the mid-gut (Fig. 7 – MG). It has six large longitudinal ridges (LR) projecting into the lumen and a few smaller ridges between them. The intima (Ii) of the ridges is 10 to 20μ thick and shows the serrations caused by the spines on its surface. The intima (Iii) also appears on the outside of the oesophageal valve. The inner layer of the epidermis (EPi) consists of roughly cubical cells while the outer layer (EPii) consists of longer, columnar cells. The circular muscle (CM) is three or four strands thick.

Discussion

According to Snodgrass (5) the proventriculus in insects, in its simplest form, is "merely the narrowed posterior end of the stomodaeum which is more or less invaginated into the anterior end of the mesenteron to form the cardiac valve". The proventriculus of the milkweed beetle is clearly of this type being indistinguishable externally from the rest of the stomodaeum, and having only a coating of small spines to distinguish it internally from the anterior part of the stomodaeum. In the genera of Cerambycidae studied by Plateau (3) it is pointed out that there is no "gésier" or proventriculus and Thiel (6) reports that in the Polyphaga in general the proventriculus is poorly developed in contrast to the proventriculus of the Adephaga, which is a definite organ in the digestive tract. Balfour-Browne (1) reports that in some Cerambycidae there is "nothing more than a thin-walled expansible crop".

Some authors have attempted to correlate the structure of the proventriculus with the type of food eaten by insects, while others have attempted to correlate it with the manner of feeding. Ris (4) maintains that the proventriculus is not well developed in insects that feed on liquids, while it is well developed in those that chew solid foods. Plateau (3), however, points out that many insects that eat solid foods have no proventriculus. Ris says that insects that have well developed proventriculi devour their food and then cease feeding, whereupon the proventriculus rechews the food. Such insects may be called "intermittent feeders" and are usually active forms and possess a large crop. In inactive insects that feed continuously (e.g. lepidopterous larvae) there is not a well developed proventriculus and the crop is small. The stomodaeum of *Tetraopes tetraophthalmus* belongs in this latter category since it consists of a small crop and a poorly defined proventricular region.

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THE EFFECT OF GONADAL HORMONES ON LIVER NUCLEIC ACIDS IN THE IMMATURE PULLET[1]

By D. G. Chapman,[2] A. A. Hanson,[3] R. H. Common,[4] and W. A. Maw[5]

Abstract

The ribonucleic acid, desoxyribonucleic acid, and the ratio of ribonucleic acid to desoxyribonucleic acid in the liver of immature pullets treated with 4.5 mgm. testosterone propionate over a 12-day period were all increased when this treatment was supplemented by treatment with estrogen. The possible significance of these effects in relation to other effects of similar gonadal hormone treatments is discussed.

Introduction

Treatment of pigeons or immature pullets with estrogen increases the weight of the liver and the weight of the liver crude protein per kgm. live weight (3, 4, 5). The magnitude of these particular effects is not appreciably influenced by concurrent small doses of androgen, even though such androgen treatment is sufficient to enhance the effects of estrogen on the oviduct and to enable estrogen to increase the rate of calcium retention (6). Evidence as to an increase of liver weight and liver crude protein during the normal transition to laying activity is less clear-cut. However, high egg production in hens has been associated with heavier livers (8). It is highly probable that the increased endogenous estrogen production associated with the onset of laying tends to produce an increase of liver weight and of liver crude protein per kgm. live weight. Increases in liver weight and liver crude protein evoked by estrogen are not merely a consequence of the increased appetite (2, 15, 16) of estrogenized birds, for estrogen produces such effects in immature pullets when the food intake is maintained equal as between estrogenized and unestrogenized pullets.

The increases in serum phosphoprotein and phospholipid that normally accompany the onset of egg laying may reasonably be ascribed to endogenous estrogen activity. Estrogens are known to evoke similar changes in the sera of immature pullets and in cockerels (4, 5) and in male, female, and castrate pigeons (11). The sera of comparable untreated immature females and males

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contain little or no phosphoprotein. Since the increase of serum phosphoprotein takes place in the absence of the ovary, the phosphoprotein is most probably synthesized elsewhere than in the ovary. If the liver is involved, then the increase in liver crude protein under the influence of estrogen might be related to the appearance of phosphoprotein synthesizing activity as well as to an increase in synthesis of phospholipid.

There is evidence (7) that tissues engaged in active protein synthesis tend to have a relatively high ratio of ribonucleic acid phosphorus (RNAP) to desoxyribonucleic acid (DNAP). Novikoff and Potter (12) have related high RNAP : DNAP ratios in chick embryos to the periods of most rapid protein synthesis. If the effects of gonadal hormones on the livers of immature pullets include an increase of protein synthesizing activity, then such livers would be expected to show an increase in the ratio RNAP : DNAP. This change might be expected even when food intake is held constant so as to rule out the effects of increased appetite. Increase of protein intake might of itself affect the ratio RNAP : DNAP (9).

The present paper deals with experiments designed to test this theory. The experiments are an extension of previous experiments on the effects of gonadal hormones on immature pullets (4, 6).

In these previous experiments it was found that estrogen supplemented with a small testosterone propionate treatment produced a closer imitation of the normal puberal changes in the pullet than did estrogen alone (6). Such small testosterone propionate treatments by themselves did not appreciably affect the liver weight or liver crude protein as compared with those of untreated pullets. In view of these results, and since the number of pullets that could be properly handled was limited, the present experiment was limited to studying the effect, if any, of estrogen plus a small testosterone propionate treatment on liver nucleic acid phosphorus as compared with the effect of the testosterone propionate alone.

Experimental

Outline of Experiment

Two groups each of 12 immature Barred Plymouth Rock pullets were used. The birds in each group were taken from the same hatching and had been reared together under the same conditions.

The birds in each group were placed at random in individual laying battery cages arranged in three blocks of four cages each. The amounts of food eaten by the different birds in a group were kept as nearly equal as possible. Control of food intake in such experiments is of fundamental importance, since gonadal hormones stimulate appetite (2, 15, 16). Moreover, nutritional factors are known to influence the RNAP : DNAP ratio of liver tissues in the rat (9, 10).

In the first group the birds were fed on a commercial chick starter. In the second group the birds were fed on the following mixture:

Ground wheat	25 lb.
Ground corn	25 lb.
Ground barley	25 lb.
Wheat middlings	5 lb.
Soybean oil meal	15 lb.
Fish meal	2.5 lb.
Salt	1.0 lb.
Calcium carbonate	2.0 lb.
Fortified fish oil (2200 I.U. vitamin A per gm.)	0.25 lb.
Manganese sulphate	6 gm. per 100 lb. mixture

The hormonal treatments were given by intramuscular injection in six equal doses on alternate days. The total amounts of oily base injected were equalized. The birds were decapitated on the second morning after the last injection and allowed to bleed as freely and completely as possible. When bleeding had stopped the livers were rapidly removed and at once placed in tared screw-capped bottles in the ice chest at approximately $-5°$ C. The livers were held in the ice chest until immediately before subsampling for analysis, and the analyses were all begun within a few hours of killing. It is essential to take all feasible measures to reduce nuclease activity in such work, since any appreciable nuclease action will introduce gross errors of observation.

Analytical Methods

The subsamples were dispersed in 10% trichloroacetic acid in a Waring Blendor. A little Hy-flo Super-Cel was added, and after further dispersion the suspended material was filtered off, washed successively with 1% hydrochloric acid and a little water, and then air-dried. Lipids were next removed by refluxing five times with Bloor's mixture (three volumes ethanol to one volume ethyl ether). The material was finally dried, weighed, and stored in the refrigerator pending analysis.

The RNAP and DNAP content of these residues was determined by Schneider's (14) method.

The difference between the total phosphorus of the residues and the total phosphorus extracted by hot 5% trichloroacetic acid was taken as a measure of the phosphoprotein phosphorus present.

Crude protein was determined by micro-Kjeldahl. Serum calcium was determined by the method of Halverson as described by Peters and Van Slyke (13). Dry matter was determined by drying to constant weight at 105° C. Phosphorus was determined by the method of Berenblum and Chain (1).

Experimental Results

The average results for all 24 pullets are set out in Table I and Table II.

TABLE I

EFFECTS OF TREATMENTS WITH GONADAL HORMONES ON OVARIES, OVIDUCTS, SERUM CALCIUM, AND LIVERS OF IMMATURE PULLETS (AVERAGES)

No. of pullets treated	6	6	6	6
Total dosage of testosterone propionate, mgm.	6 × 0.75	6 × 0.75	6 × 0.75	6 × 0.75
Total dosage of estradiol dipropionate, mgm.	6 × 0	6 × 1.0	6 × 2.0	6 × 4.0
Initial live weight, kgm.	1.01	0.97	1.05	1.08
Final live weight, kgm.	1.16	1.15	1.20	1.22
Ovary, gm.	0.30	0.28	0.26	0.25
Ovary, condition	Quiescent	Quiescent	Quiescent	Quiescent
Oviduct, gm.	0.27	16.8	18.9	21.2
Serum Ca, mgm./100 ml.	13.2	33.9	87.8	117.5
Liver weight, gm.	21.0	27.0	29.2	32.9
Liver, gm. per kgm. live weight	18.1	23.7	24.5	26.9
Liver crude protein, %	19.2	18.8	17.9	18.3
Liver crude protein, gm. per kgm. live weight	3.4	4.5	4.4	5.0

TABLE II

EFFECTS OF TREATMENTS WITH GONADAL HORMONES ON LIVER NUCLEIC ACID PHOSPHORUS OF IMMATURE PULLETS. AVERAGES FOR SAME BIRDS AS IN TABLE I

No. of pullets treated	6	6	6	6
Total dosage of testosterone propionate, mgm.	6 × 0.75	6 × 0.75	6 × 0.75	6 × 0.75
Total dosage of estradiol dipropionate, mgm.	6 × 0	6 × 1.0	6 × 2.0	6 × 4.0
RNAP, mgm. per 100 gm. fresh liver	55.6	57.6	61.8	59.6
Liver RNAP, mgm. per kgm. live weight	10.0	13.8	15.2	16.1
DNAP, mgm. per 100 gm. fresh liver	32.4	28.4	26.0	23.8
Liver DNAP, mgm. per kgm. live weight	5.83	6.73	6.35	6.40
Average RNAP, mgm. per 100 gm. fresh liver	1.72	2.03	2.38	2.50
Average DNAP, mgm. per 100 gm. fresh liver				
Liver RNAP, mgm. per kgm. live weight	1.72	2.05	2.40	2.52
Liver DNAP, mgm. per kgm. live weight				
RNAP, mgm. per 100 gm. liver crude protein	290	306	345	326
DNAP, mgm. per 100 gm. liver crude protein	169	151	145	130
Phosphoprotein P, mgm./100 gm. fresh liver	1.5	4.0	5.0	9.0

The data in Table I for ovary weights, oviduct weights, serum calcium, liver weights, and liver crude protein are in agreement with results previously reported (4, 5), and serve to define the state of the pullets.

The data for RNAP and for DNAP per kgm. live weight were analyzed statistically in order to measure the significance of the effects of estrogen. The possibility that initial live weights might have influenced these results was examined by covariance. The covariance test showed that differences in initial live weights did not account for the treatment effects under the given experimental conditions.

The data for RNAP and for DNAP per kgm. live weight were subjected to an analysis of variance. The main features of the analysis are presented in Table III.

TABLE III

ANALYSIS OF VARIANCE OF DATA FOR RNAP AND DNAP PER KGM. LIVE WEIGHT

Source of variance	D.f.	Mean squares	
		RNAP, mgm. per kgm. live weight	DNAP, mgm. per kgm. live weight
Treatments:	3		
Estrogen vs. no estrogen	1	112.001**	1.967**
Single vs. double and quadruple dose estrogen	1	13.813*	0.513
Double vs. quadruple dose estrogen	1	2.901	0.008
Groups (i.e. experiments)	1	114.407**	4.770**
Treatments × groups	3	9.905*	0.555
Blocks	4	12.132*	0.942**
Remainder	12	2.348	0.162

**Significant at 5% level.*
*** Significant at 1% level.*

Estrogen treatments increased the liver RNAP per kgm. live weight to a highly significant degree. The double and quadruple dosages of estrogen produced a significantly greater amount of liver RNAP than the single dosage. The quadruple dosage did not, however, produce a significantly greater amount of RNAP than the double dosage. It may be remarked that the difference between estrogenized pullets and those receiving no estrogen remains significant when compared with the mean square for treatments × groups.

The estrogen treatments also evoked a highly significant increase in liver DNAP per kgm. live weight, but the differences between birds on different levels of estrogen were not significant in this case. Even in the case where estrogen increased the amount of liver DNAP per kgm. live weight, therefore, a relatively greater increase was brought about in liver RNAP.

Discussion

The data now reported prove that when estrogen treatments are superimposed upon androgen treatments sufficient to evoke puberal comb and wattle changes in sexually immature pullets, then under the given conditions the

estrogen (*a*) increases total liver DNAP, but to a limited extent; (*b*) increases liver RNAP; and (*c*) increases the ratio RNAP : DNAP in the liver.

It is already known (4, 5) that the increase of liver crude protein per kgm. live weight evoked by estrogen is not seriously modified by concurrent administration of amounts of testosterone propionate that are adequate to produce puberal comb and wattle changes and to enhance the effects of estrogen on hypertrophy of the oviduct. When the present results (*vide* Tables I and II) are examined in the light of this observation, then it would seem that estrogen changes the liver protein qualitatively as well as quantitatively. The total liver DNAP is increased by estrogen, but the amount per 100 gm. liver crude protein falls. The total liver RNAP and the amount per 100 gm. liver crude protein both increase. The amount of liver RNAP + DNAP per 100 gm. liver crude protein displays a remarkable tendency to constancy.

Before proceeding to a tentative interpretation of these results, however, it must be recalled that the livers were not perfused and that reliance was placed on free bleeding after decapitation to free the livers from appreciable remaining amounts of blood. (Perfusion will ensure removal of blood, but it was considered more desirable to avoid the probability of extensive nuclease action during prolonged perfusion than to remove the last traces of blood.) Estrogen evokes marked changes in blood composition. One cannot, therefore, completely dismiss the possibility that the differences in RNAP and DNAP may be a reflection of differences in amounts of blood remaining in the livers. Conclusive evidence on this point is still lacking, but the liver material seemed remarkably free from blood during preparation.

Conclusions

Pending the detailed investigation of residual blood and of nuclease action in the livers of such experimental pullets, therefore, the following tentative conclusions may be drawn from these experiments:

(1) Treatment of the immature pullet with estrogen combined with androgen increased the amount of liver crude protein per kgm. live weight, in confirmation of previous observations. These increases have now been found also to modify the relative proportions of ribonucleoprotein and desoxyribonucleoprotein in the liver. The effects are independent of any stimulatory effect of estrogen on appetite and protein intake.

(2) The modifications were such as would be expected from an increase in protein synthesizing function or the acquisition of new protein synthesizing functions. Increasing doses of estrogen increased the total amounts of liver RNAP and DNAP and also increased the ratio RNAP : DNAP in the liver. The total amount of RNAP plus DNAP per 100 gm. liver protein remained relatively constant.

(3) It may be suggested tentatively that endogenous gonadal hormone activity will tend to evoke similar changes in the liver when reproductive activity begins normally.

Acknowledgments

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EXCHANGE OF MATERIALS IN A LAKE AS STUDIED BY THE ADDITION OF RADIOACTIVE PHOSPHORUS[1]

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Abstract

On July 1, 1948, 100 millicuries of radioactive phosphorus, P^{32}, was added to the surface of an acid bog lake of area 0.3 hectares (0.8 ac.) and depth 6 m., situated near Halifax, Nova Scotia. The lake had a well developed zone of cold, stagnant water at the bottom with a thermocline above. Within a few hours the P^{32} was taken up strongly by the sphagnum (which made up most of the lake margin), by the floating plankton, and by sponges. Within a few days it had reached the bodies of fishes (*Fundulus* and *Notemigonus*), and within a fortnight had accumulated in their skeletons and viscera. No appreciable uptake of P^{32} by such bottom rooted forms as the cranberry (*Vaccinium*) and leather leaf (*Chamaedaphne*) was observed for more than two weeks. Penetration of added phosphorus into the lake depths was very slow, and even at the end of eight weeks the material was only doubtfully detected at the bottom. There was no evidence of active uptake by bottom mud. The ratios of maximum count per wet weight of organism to maximum count for the same quantity of lake water were as follows:

Zooplankton	40,000
Fundulus	13,000
Sponge	4500
Sphagnum	400
Vaccinium	300
Blue-green alga	300
Chamaedaphne	100
Nuphar	80

One morning during the summer of 1948, 100 millicuries (mc.) of radioactive phosphorus, P^{32}, was added to a small lake in the vicinity of Halifax, Nova Scotia. The purpose of this paper is to describe the utilization of the phosphorus by plants and animals and its penetration into the water. The experiment was essentially similar in technique to familiar tagging experiments in which selected animals are marked in some way, then released. When the animals are taken again, the migration is evident.

The Lake

The lake in question is situated 12 mi. from Halifax, 100 yd. from the St. Margaret's Bay Road. It occupies an area of about 0.3 hectares (0.8 ac.), and contains some 16,000 cu. m. of water. The lake is fed by an inlet supplying approximately 4 cu. m. per hour during the summer season, while the outlet was observed to carry away some 5.5 cu. m. per hour, the difference being presumably due to seepage.

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Contribution from the Departments of Chemistry and Biology, Dalhousie University, Halifax, N.S., and the Fisheries Division of the Nova Scotia Department of Trade and Industry.

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Botanical characteristics of the lake are indicated in Fig. 1, which gives a typical picture of an acid bog lake, filling in by encroachment from without. The bordering region is a heath association, largely *Chamaedaphne* and

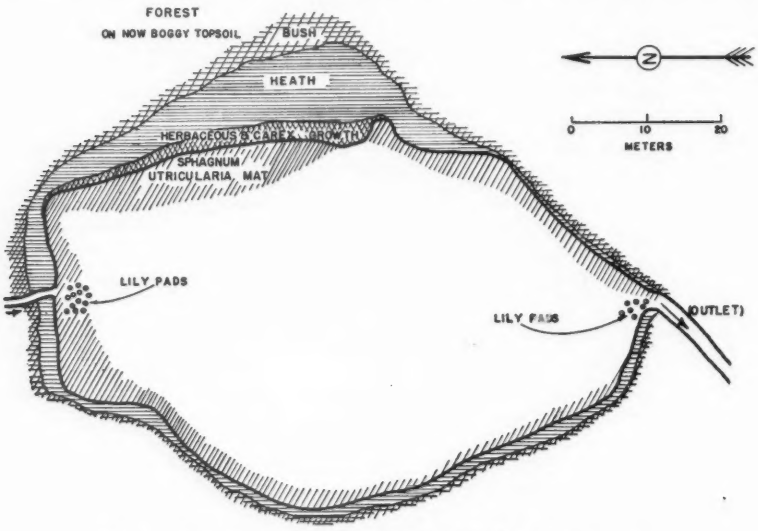

FIG. 1. *Botanical characteristics of the lake used for experiments.*

Myrica Gale, that gradually merges into the sphagnum margin of the open water. Ninety per cent of the margin consists of a wide mat of sphagnum, which, being covered with heath growth, produces a springy mat that is gradually filling in the lake. The fundamental shape of an acid bog lake is that of the bulb of a thistle tube, the area being greater some distance below the surface than at the surface.

Although the lake is very small and its description seems to answer to that of a pond, its depth varies from 3 m. near shore to 7 m. at its deepest part. There was a thermocline and hypolimnion, which are shown in Fig. 2. The thermocline extended down for some three meters, below which the temperature was only some 5° C. throughout the summer.

Fig. 3 shows the oxygen characteristics of the lake. At no part was there saturation with oxygen and a marked gradient existed so that below three meters oxygen was very scarce, and below five meters undetectable. A peak in the oxygen curve at a depth just below one meter is not easy to explain, but has been observed in a number of lakes in this region.

The oxygen and temperature characteristics of the lake are such that the depths had too little oxygen for trout, while the surface waters in summer might be uncomfortably warm. At any rate no trout were observed in the lake during the experiment. The only two native fishes taken were *Fundulus diaphanus* and *Notemigonus crysoleucas*. As these fishes were in scarce supply

and as it was desired to obtain samples at intervals, some four thousand *Fundulus* were seined from neighboring lakes and introduced into the experimental area a few days before the addition of P^{32}.

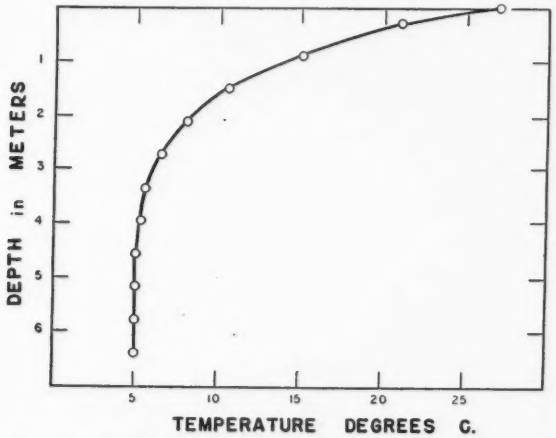

FIG. 2. *Variation in lake temperature with depth. Observations were made in the morning and show a thermocline and hypolimnion. Doubtless a series of observations made in late afternoon on a sunny day would show an epilimnion as well.*

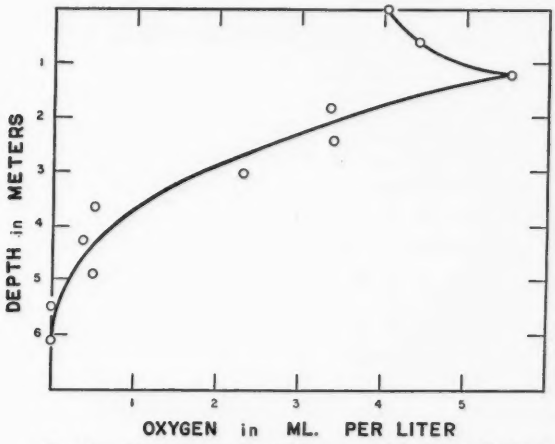

FIG. 3. *Variation in dissolved oxygen of the lake with depth.*

Methods

A quantity of P^{32} having a stated activity of 100 mc. on June 25, 1948, at 2.00 p.m. was obtained from the laboratories of the National Research Council. At the time of its introduction into the lake the presumed activity was about 76 mc. This material, dispersed in 27 gm. of potassium dihydrogen phosphate was dissolved in water in a glass container of about 25 liters capacity. The

method of solution, in order that the greatest precaution be taken, consisted of lowering the vial of P^{32} on a cord into the larger vessel of water, the contents of the vial then being dissolved by pumping a continuous stream of water into it with a syringe bulb and tubing. The large container of active phosphate was placed on a platform on a rubber boat and pushed back and forth across the entire surface of the lake by another boat, from which was operated a syringe bulb that pumped air into the jar and started a siphon by means of which active liquid was delivered into the water. Propulsion provided by an outboard motor served to mix the surface water thoroughly. The method allowed the active material to be well isolated, the vessel being situated about 10 ft. from the man controlling the siphon. Deposition required about one hour and was completed on July 1 at 7.00 a.m., which is called "zero hour". The sun was shining, and the water dead calm. The technique of deposition was tried out by a dummy run on the day previous to the actual experiment.

Total phosphorus in the lake was measured before the experiment, by the method of Robinson, using a Lumetron photoelectric colorimeter. Surface values averaged 25 parts per billion and those in deeper water 15 p.p.b. Thus the lake of 16,000 cu. m. would contain some 320 gm. of phosphorus, and 27 gm. of potassium dihydrogen phosphate, containing 6 gm. of phosphorus, would increase the natural store by 2%. If we take the inorganic phosphorus to be about 1/10th of the organic in this type of lake, it might be that the enhancement of inorganic phosphorus amounted to some 20%. However, the ratio of organic to inorganic phosphorus is not accurately known, the latter being insufficiently concentrated for accurate estimation, as is the case in most Halifax County lakes.

Samples of lake water were collected at various depths using a Foerst Water Bottle. Mud samples were taken with an Ekman dredge. Lake temperatures were measured with a Leeds and Northrup "Thermohm" Electric Resistance Thermometer. Oxygen estimations were made by the Winkler method. Plankton samples were collected in a conventional silk net.

The methods for determining the quantity of P^{32} in the water consisted of evaporating the sample (100 ml. in initial tests) to about 3 ml. using gas burners and fans under a fume chamber. Evaporation to dryness was carried out in aluminum counting trays on hot plates. As the activity in the water decreased, larger samples were evaporated, 900 ml. being the largest amount used. The trays were then placed in a lead castle and counted with Geiger tube and scaler. Evaporation was, for several reasons, more satisfactory than precipitation with sulphate–molybdate, which was also tried. Precipitation and evaporation methods checked satisfactorily.

Fish were prepared for the phosphorus counts by ashing in a perchloric acid and nitric acid mixture. The phosphorus was precipitated with sulphate–molybdate reagent, filtered, and counted on the filter papers.

The plankton samples were collected in a Büchner funnel on discs of filter paper cut to the correct size to fit a counting tray. The dried material was then placed directly in the counter.

Sphagnum was first dried, then ground with mortar and pestle, and subsequently spread evenly over tared counting trays. After weighing, the position of the sphagnum was fixed with label glaze.

Sponges, and higher plants, were treated in essentially the same way as sphagnum.

Four counters and scalers were used, being brought into relation with each other by counts on a standard phosphorus sample. For a number of reasons variations occur in all counters from day to day. Allowance was made for these by daily counts of standard uranium samples. Conditions of geometry that prevailed at the time of standardization of the four counters were maintained throughout the experiment. The sample thickness in no case warranted the application of a correction for self absorption.

In all the statements of counts and in the illustrations in this paper, corrections for decay have been applied in such a way as to bring the P^{32} to the value that it had at the time it was standardized before shipment. No additional standardization was carried out, it being presumed that the quantity of P^{32} was as accurately known as other major variables in the experiment, such as the lake volume or the amount of natural phosphorus in the lake.

The method for converting counts of the samples into counts per minute per 100 gm. of material as used on the graphs was as follows:—Consider the point on Fig. 7 for 28 hr. after zero hour. The plankton sample was taken from the lake at 11 a.m. on July 2 and counted at 8 p.m. on July 5 with Geiger–Müller tube No. 3. The dry weight of such a sample was 0·0698 gm. The ratio of dry weight to wet weight was 4.5. The counts per minute on the sample were 4585 and the background was 11 counts per minute. The uranium standard gave 9404 as compared to 9660, which had been taken as a standard count on standard day. At 8 a.m., July 5, only 60% of the original activity was left, which meant that the count must be multiplied by 100/60 in order to bring the count up. The factor relating counter No. 3 to counter No. 1 (standard counter) was 2.384. The corrections thus applied give the figure used on graph from the following expression:

$$\text{Counts per minute} = \overset{(a)}{(4585 - 11)} \times \overset{(b)}{\frac{9660}{9404}} \times \overset{(c)}{\frac{100}{60}} \times \overset{(d)}{2.384} \times \overset{(e)}{\frac{100}{0.0698 \times 4.5}} = \overset{(f)}{5.95 \times 10^6}$$

(a) = original count on counter
(b) = background
(c) = ratio correcting for fluctuation in counter
(d) = correction for decay
(e) = factor relating counter No. 3 to standard counter
(f) = conversion to 100 gm. wet weight
(g) = c/m as seen on Fig. 7.

Radioautographs were made by pressing the dried or frozen samples against a piece of high-speed dental X-ray film, first placing a piece of waterproof plastic between the sample and the emulsion. The fish were frozen and sagitally sectioned leaving, however, the vertebral column intact. Such specimens were kept frozen during the whole exposure time. Exposure times were empirical and, in general, lasted from 1 to 15 days, depending on the concentration of P^{32}.

Penetration of P^{32} into the Depths

Analyses of mud from the lake bottom were made from time to time, but no appreciable concentration of P^{32} was observed. Water samples were taken at a station over the deepest part of the lake, the counts being shown in Fig. 4.

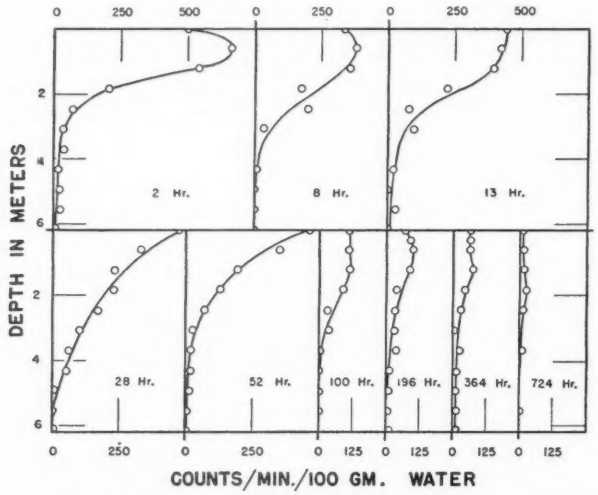

FIG. 4. *Counts per minute of P^{32} at different depths and at increasing intervals after the time of addition. Each point on the curve represents one determination. All counts were made at one station over the deepest part of the lake.*

From very early in the series some counts of phosphorus were found in the deep waters of the lake, although these were so small compared with surface counts that they might be due to water carried down with the depth sampling bottle. The first evidence of penetration below the thermocline was observed at 28 hr. At no time up to the end of observations was the P^{32}, added at the surface, clearly detected in the deepest water.

One further feature of Fig. 4 deserves mention, namely, a peak below the surface that is first noticed at 196 hours. This peak is believed to be caused by a runoff of surface water in the lake. It might be expected that such a peak would gradually move down into the depths of the lake as warmer water was removed from the surface, and this is what seems to be occurring in the two final readings.

There are two reasons why added phosphorus could appear in the depths of the lake, one of them being turbulence, and the other being due to accumulation of P^{32} in the bodies of plankton organisms at the surface. Plankton exhibits diurnal vertical migratory behavior and could therefore take phosphorus to the bottom. Dead plankton organisms might also sink to the bottom giving up their phosphorus in the deeper layers of water.

Fig. 5 is an attempt to follow the fate of the added phosphorus as it disappeared from the lake. The horizontal line at the top is the theoretical value for counts, of the initial addition of 100 mc. of P^{32}. The curve (or

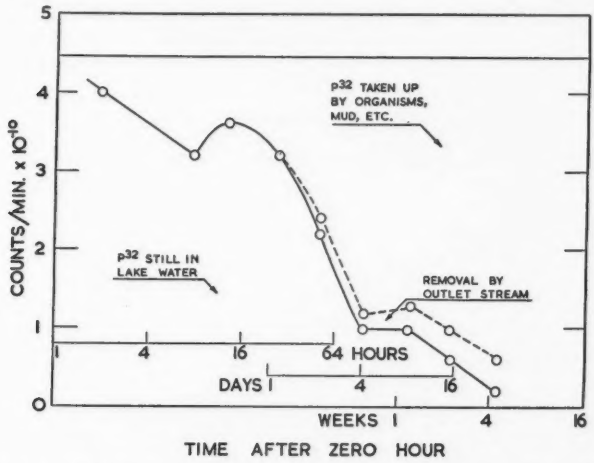

FIG. 5. *Fate of the phosphorus added to the lake. Horizontal line at the top is the theoretical value for counts of the initial addition of 100 mc. of P^{32}. The points on the curve, or lower curve (solid line) are obtained from the data plotted in Fig. 4. Take for instance, the two hour curve of Fig. 4, and draw horizontal lines through each point, to divide the lake into a number of layers, each 0.61 m. (2 ft.) thick. For each layer take as the P^{32} concentration, the average of the two points above and below it. Now from a contour map of the lake (not published) the volume in each layer is established. The total counts in each layer are:*

$$\frac{\text{Average of two points in Fig. 4} \times \text{total ml. water in layer of lake}}{100}$$

Summing up the values for all 10 layers one obtains the total number of counts in the lake at two hours, which is set down as the initial point in Fig. 5. It might be noted that readings for the deeper layers were often negligible, because P^{32} was scarce and the contours closing in. If the lake has overhanging margins as suggested above, a constant volume of water under the margins would be overlooked in calculations. No allowance has been made for this.

The upper curve (dotted line), which has the lower curve as its base, shows the cumulative discharge of P^{32} by the outlet stream.

lower curve) gives the total disintegrations in the lake. The upper curve (which has the lower curve as its base line) shows the cumulative discharge of the outlet stream. Thus the upper block of the diagram represents the uptake by organisms, etc., within the lake. It is apparent that four-fifths of the added phosphorus was utilized, largely by organisms, within three weeks.

The rapid disappearance of the P^{32} is rather disturbing to anyone thinking of adding fertilizer to natural waters as a fish cultural procedure. In the present case the increase in the phosphorus content of the water was only 2%, and it might be thought that if a great deal more had been added, there would have been enough to satisfy the sphagnum and fish and still leave the lake with a high phosphorus value for some time. General experience with added nutrients does not, however, support such an assumption. We have, for example, added massive amounts of phosphorus, in the form of basic slag, to acid bog lakes in the vicinity of the one here under discussion. Basic slag was selected because the phosphorus in it is liberated rather slowly, and it was hoped that a continuing supply would be given off. In so far as the hope was realized, the effect would be to hold up the phosphorus level of the lakes. Some results are given in Table I, from which it is apparent that neither the amount of phosphorus initially present in the lake, nor the amount added, stands in any clear relation to the rate of removal.

The lakes mentioned in Table I are shallow, of 5 to 10 ac. in area, and with a negligible runoff in summer. In all cases the theoretical amount of added

TABLE I

DISAPPEARANCE OF PHOSPHORUS WHEN ADDED TO ACID BOG LAKES. ANALYTICAL VALUES ARE IN MGM. PER CU. M. OF SURFACE WATER

Lake	Phosphorus in lake at start	Maximum observed, one or two days after addition	Phosphorus present after one month	Percentage of added phosphorus that disappeared in one month
South Weaver (1st addition)	15	75	45	50
South Weaver (2nd addition)	45	230	45	100
Holland (1st addition)	70	230	150	50
Holland (2nd addition)	150	475	230	75
This paper (P^{32})	20	—	—	90

phosphorus that could go in to solution was 450 mgm. per cu. m. This value is based on the immediate solubility of the phosphorus in basic slag in water of pH 5.5 and, as the table shows, was not reached in any case.

Uptake by Sphagnum

Estimations at three stations on the margin of the lake were made from time to time, of P^{32} in sphagnum at the surface, and one-half meter below the surface, the results being shown in Fig. 6. It will be noticed that the base line of Fig. 6 has been drawn logarithmically, and the same is true of the

curves to follow. Observations of sphagnum and other materials were made at logarithmically spaced time intervals so that the distances between the operations shown on Fig. 6 and other figures are approximately equal.

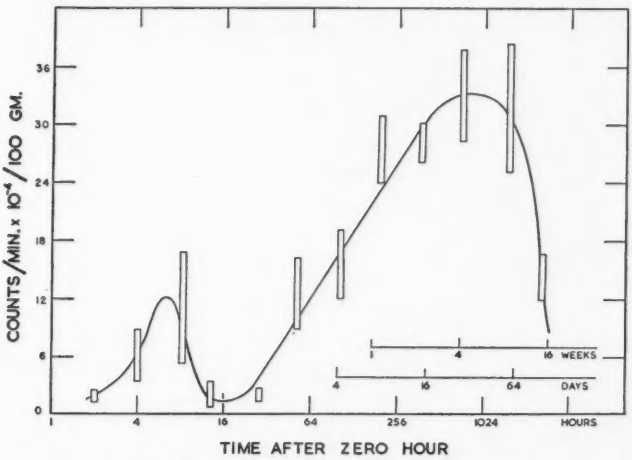

FIG. 6. *Variation in P^{32} counts in sphagnum from the lake. Observations were made at three stations on the lake margin, at each of which one sample of sphagnum was taken at the surface and one 0.5 m. down. Results of the six observations were averaged and the standard error calculated. Each vertical line on the figure shows the limits of the standard error of a mean.*

The vertical lines on Fig. 6 show the limits of the standard errors of the means, as will be clear from an example. Take the readings at approximately four days (100 hr.).

Marginal station	Depth	Counts per minute per 100 gm. sphagnum $\times 10^{-4}$
A	Surface	10.3
A	0.5 m.	9.6
B	Surface	13.0
B	0.5 m.	7.0
C	Surface	26.0
C	0.5 m.	28.0

The average count was 15.7 and the standard error 3.7. Hence the limits were 19.4 and 12.0, between which the line has been drawn on Fig. 6.

The curve of Fig. 6 appears to exhibit two peaks, the validity of the first of which must be considered. It will be noticed that the first and fourth observations were low and the second and third were high. The difference between the means of the first and second observations is scarcely significant, and the same is true of the third and fourth. However, if we combine the second and third on the one hand and the first and fourth on the other, the difference becomes significant. We have therefore placed an initial peak in the curve.

It is interesting to note that the first peak in the sphagnum curve corresponds in time to a trough in the water curve of Fig. 5, and the subsequent trough in the sphagnum curve corresponds to a peak in Fig. 5. If it could be assured that a fall in sphagnum P^{32} caused a measurable rise of the lake P^{32}, it would be possible to determine the mass of participating vegetation. Further investigations under controlled conditions are in progress along these lines.

A glance at Fig. 6 shows that the uptake by sphagnum exhibited a second peak at about three weeks. It appears reasonable to suggest that sphagnum possesses two mechanisms of phosphorus uptake, the first of which might be due to physical processes unconnected with active cell metabolism. The second peak might be due to the incorporation of phosphorus into the protoplasm in some permanent way.

The decline after the second peak could be attributed to two causes. First, a loss of phosphorus could occur from the sphagnum back into the water. Second, it would be expected that the initial mass of cells containing P^{32} would be increased by the addition of new cells with diminishing quantities of P^{32}. This would have the effect of diluting the sample taken for analysis.

No significant difference was observed between the surface counts and those taken 0.5 m. down.

Plankton

Samples of plankton were collected from time to time, the analysis of results being shown in Fig. 7. No attempt was made to separate plankton

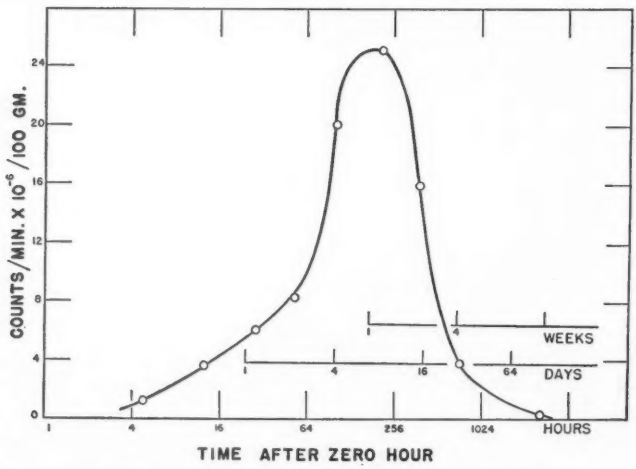

FIG. 7. *Uptake of P^{32} by zooplankton, which consisted almost wholly of Diaptomus.*

into species or groups, but this, as a matter of fact, would not have improved the results because the plankton as collected turned out to be practically a pure culture of the copepod, *Diaptomus*. Fig. 7 does not exhibit a preliminary

peak like that shown by sphagnum, but rises gradually to a single maximum at about 200 hr., after which the curve declines steadily. It should be noted that the situation as regards plankton is quite different from that of sphagnum, the former being composed of animals whose lives are of short duration so that the counts shown in Fig. 7 do not represent the conditions in a single generation of forms that live through the summer, but in several generations.

The decline in Fig. 7 reflects merely the fact that there was less P^{32} in the water to be picked up by new generations of plankton at the time the later observations were made.

Fish

The curve describing fish, which covers observations on both *Fundulus* and *Notemigonus*, is given as Fig. 8. It will be noticed that no P^{32} could be found

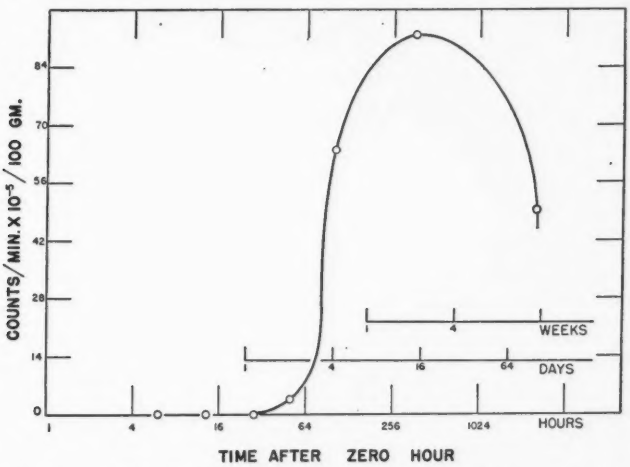

FIG. 8. *Uptake of radioactive phosphorus by two fishes, Fundulus and Notemigonus. Each point from analysis of one specimen.*

in the fish until some 50 hr. after the beginning of the experiment. Such a late beginning of deposition in fish contrasts strikingly with the early uptake observed in the plankton and suggests that a different mechanism may be operating in the two cases. In the case of the plankton it is easy to imagine that inorganic phosphate is taken up directly, while in the fish the time lag would suggest that the P^{32} must first be taken up by the plankton organisms and incorporated into their tissues. The fish would then receive phosphorus by eating the plankton.

Obviously there is a considerable quantity of water swallowed by the fish. It will also be remembered that there is a continuous stream of water, going in through the gills by osmosis, which is eliminated by the kidneys. Despite this, however, there is apparently no selective storage of phosphorus in the fish tissues directly from the water.

The curve for fish exhibits a decline, with the peak at approximately the same place as for sphagnum, and the causes of the decline may be the same as given for sphagnum.

Other Organisms

As contrasted with the results already described, P^{32} was not observed in the leaves of marginal rooted organisms until a fortnight after the experiment began. Fig. 9 shows the counts on leaves of cranberry and leather leaf, both making up heath at the lake margin and collected by reaching out of a boat. The delayed appearance suggests that P^{32} reached the leaves via the roots. It may be presumed that P^{32} was first taken up by the roots and later reached the leaves. The time required for appearance in the leaves may be made up of (*a*) time to reach the roots, perhaps considerable; (*b*) time to penetrate the roots, perhaps very short; (*c*) time for translocation to the leaves. The difference in shape between the two curves of Fig. 9 may be related to the fact that the time of flowering and fruiting for *Chamaedaphne* was just over, while that for the cranberry was just starting.

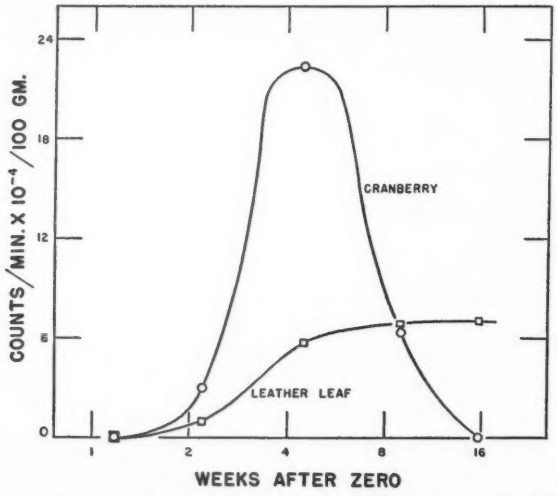

FIG. 9. *Concentration of P^{32} in the leaves of two higher plants at intervals after deposition. Both the leather leaf (Chamaedaphne) and the cranberry (Vaccinium) were rooted in the marginal bog.*

A further observation, which may be mentioned at this point, was made on the growing tips of *Hypericum*, the St.-John's-wort, which was also growing at the lake margin. Forty-eight hours after zero, the count per 100 gm. was 4×10^5, a somewhat higher value (two- to tenfold) than was ever recorded for the leaves of higher plants.

Fig. 10 illustrates the uptake of P^{32} by two additional organisms, yellow water lily leaves (*Nuphar*), and sponge. In these cases our observations began late (four days) so that the initial time of uptake cannot be precisely

stated. The general structure of a sponge would lead one to expect an early uptake, while, by contrast, the water lily leaves would presumably have to obtain P^{32} via the roots, and hence exhibit some delay. The peak on the

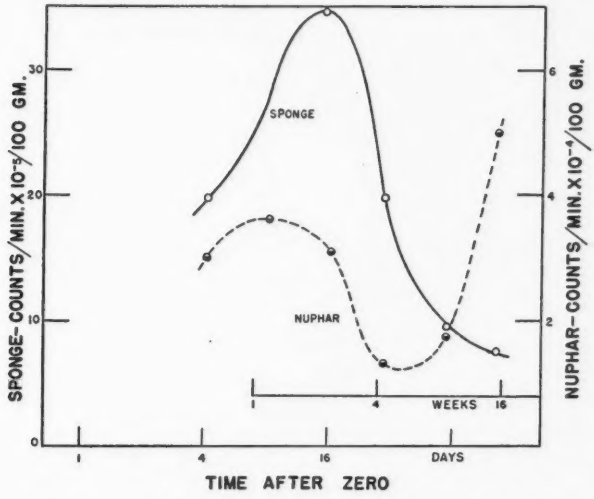

FIG. 10. *Amount of added phosphorus present in the sponge (Spongilla) and yellow water lily (Nuphar) at various times.*

sponge curve might have the same cause as given above for sphagnum. It would be premature to attempt an interpretation of the water lily variations.

Four counts on the alga *Batrachospermum* were also made between nine days and eight weeks from zero time. No trend was discernible, the average value being about 6×10^5 counts per 100 gm. By the time the first count was made the maximum value was already established. It is reasonable to think that such a form would take phosphorus up quickly, but proof is not at hand.

Comparative Uptake

It is of interest to measure the intensity of phosphorus assimilation by the various plants and animals in the lake. There are two ways of making such a calculation as shown in Table II.

First, we may give the absolute uptake, which is the ratio of the maximum observed value for an organism (peaks of curves) compared to the maximum value for the same weight of water (Fig. 4, subsurface at two hours). It is seen that animals take up more than plants; higher animals more than lower animals; and lower plants more than the higher plants. As regards the last statement, however, it must be remembered that the measurements on whole algae and on all exposed parts of sphagnum are not strictly comparable to the leaf counts made on higher plants. It was noted above that the growing tips of a higher plant gave greater counts than leaves.

TABLE II

COMPARATIVE UPTAKE OF P^{32}

Common name	Organism	Absolute uptake $\times 10^{-2}$. Ratio of maximum counts per 100 gm. organism to maximum counts per 100 gm. water	Relative uptake $\times 10^{-7}$. Maximum counts per min. per gm. total phosphorus in organism
Alga	*Batrachospermum*	10	660
Sphagnum	—	4	10
Cranberry	*Vaccinium Oxycoccus*	3	3
Leather leaf	*Chamaedaphne*	1	1
Yellow water lily	*Nuphar*	1	2
Sponge	*Spongilla*	45	500
Zooplankton	*Diaptomus*	400	13
Fish	*Fundulus* and *Notemigonus*	130	2

A second calculation gives the relative uptake, by answering the question: how much P^{32} can be taken up in proportion to the total phosphorus content of the organism? Fish will serve as an example of the method of calculation. The peak of the curve in Fig. 8 is observed to have a value of 91×10^5 counts. By conventional analysis fish were found to contain 0.56% of total phosphorus.

$$\text{Thus } \frac{91 \times 10^5}{0.56} = 1.62 \times 10^7,$$

which has been set down as the nearest whole number, 2, in Col. 4 of Table II. The ratios in Col. 4 provide a comparison of the rate of turnover of phosphorus by each plant or animal. Maximal values are observed for alga and sponge, the simplest forms dealt with. Next, but greatly lower, come sphagnum and *Diaptomus*. Fish and higher plants made up a still more sluggish group. The high value for *Diaptomus* as compared to fish is, perhaps, related to (*a*) the short life of a generation of plankton organisms, making a rapid turnover of material necessary, and (*b*) the mass of inorganic phosphate in a fish skeleton, which is renewed fairly slowly (see further on).

In both columns of the table the sphagnum ratios are below some of the others. Nevertheless, sphagnum is believed to be the main remover of P^{32} in this lake, because of its great abundance.

Radioautographs

Radioautographs of a number of organisms were made from time to time with a view to determining the site of deposition of P^{32}. Fig. 11 is a picture of a sponge, *Spongilla*, taken from the lake four days after the beginning of the experiment, exposure time being 21 hr. A general darkening of the whole tissue is evident.

Fig. 12 is a frog that had been in the lake for 40 days, and was exposed to the film for 15 days. The phosphorus has become deposited in the skeleton and is quite clearly shown in the jaws and the limbs. The variations of

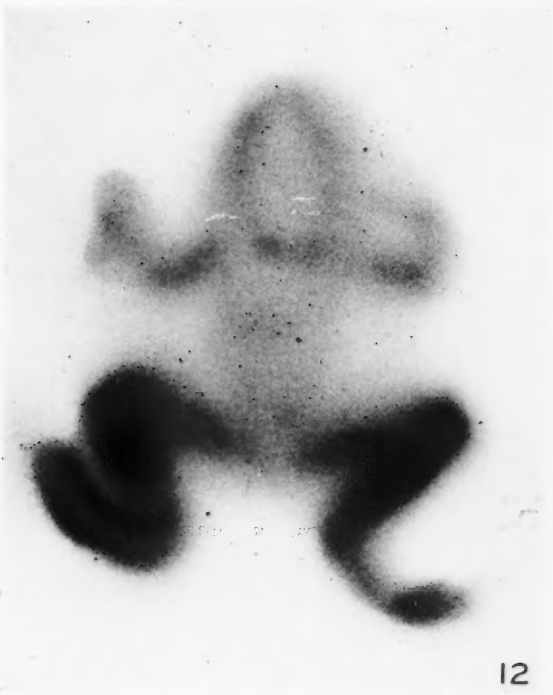

FIG. 11. *Radioautograph of a sponge taken from the lake four days after addition of radioactive phosphorus. Exposure, 21 hr.*

FIG. 12. *Radioautograph of a frog taken from the lake 40 days after addition of P^{32}. A concentration of material in the skeleton is evident. Exposure, 15 days.*

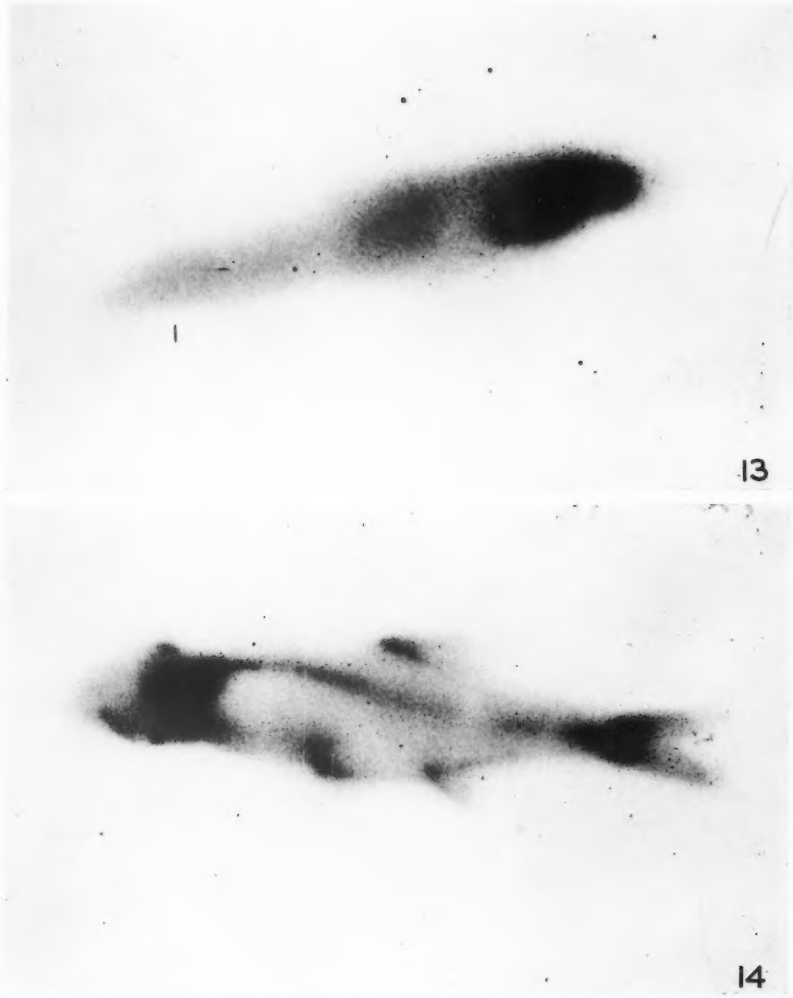

FIG. 13. *Radioautograph of Fundulus taken from the lake nine days after the experiment started. The phosphorus appears to be distributed over the whole body, with some concentration in the viscera. A dark spot might be the liver. Exposed six days.*

FIG. 14. *Distribution of P^{32} in Notemigonus removed 16 days from the start. Concentration of the new phosphorus in the axial skeleton and at the bases of the fins is evident. Exposed 24 hr.*

intensity of darkening between fore and hind limbs do not indicate differences in phosphorus concentration, but the proximity of the parts to the photographic film.

Fig. 13 is a specimen of *Fundulus* taken seven days after the beginning of the experiment and exposed for six days. There is evident a general darkening over the whole body with some tendency for a concentration of P^{32} in the region of the gut. Possibly a heavily pigmented spot in the anterior gut region might represent the liver.

Fig. 14 is a specimen of *Notemigonus* taken 15 days from the start, and exposed for 24 hr. A considerable change is noticed from the preceding picture, for by this time the phosphorus had begun to show a marked concentration in the skeleton including both the vertebral column and the bases of the fins and tail.

Conclusions

1. P^{32} uptake by plants and zooplankton is a matter of minutes or hours, not days or weeks.
2. Sphagnum appears to have two mechanisms of phosphorus uptake.
3. Zooplankton can apparently either take up inorganic phosphorus directly, or feed with great rapidity on microorganisms that have absorbed the added phosphorus at once.
4. Fish evidently cannot take up inorganic phosphorus directly, but presumably obtain it by feeding on plankton.
5. Phosphorus taken up by fish is initially dispersed through the body generally, with some concentration in the gut tract area. Later it is segregated into the skeleton, both axial and appendicular.
6. Living organisms can concentrate added phosphorus to a level up to 40,000 times that of the water.
7. Estimations of soluble phosphorus do not appear to provide a reliable guide to the level of fertility, at least in this type of lake. Added phosphorus is quite rapidly removed. We may picture the phosphorus level at equilibrium, as determined by the tendency of plants (in proportion to their abundance) to pull phosphorus out of the water, balanced by replacement from decaying plants and by the inlet. Thus the same equilibrium level for phosphorus might be reached under a variety of conditions. There may, however, very well be a quantitative relation between the rate of removal of added phosphorus and plant abundance.
8. Fertilization of acid bog lakes with a view to fish production, appears to be relatively unprofitable, for the fertilizer will quickly be incorporated into the sphagnum. Most of the phosphorus in sphagnum goes to form peat although some may be later returned to the lake water.

Acknowledgments

From the standpoint of equipment, this work was made possible first by the fact that the National Research Council placed in the Chemistry Department of Dalhousie University the counters and scalers necessary to make estimations of P^{32} and second, that the Nova Scotia Department of Trade and Industry, through its Inland Fisheries Survey, provided the necessary field collecting and measuring equipment.

We are indebted to Mr. James Lewin of the Inland Fisheries Survey for surveying the lake, and for much assistance with the field work. Miss Shirley Mason conducted a botanical examination of the lake, and Messrs. D. A. Livingstone and M. L. Cameron also aided with the field work. Messrs. I. H. S. Henderson and I. I. Tingley assisted with the preparation and analysis of samples. The advice of Dr. W. J. Chute and Dr. H. P. Bell was frequently sought, and very generously given.

Without the assistance of friends, particularly those mentioned, it would have been impossible to carry through the work.

DISTRIBUTION OF P^{32} IN CHICK EMBRYOS[1]

By J. B. O'Neil,[2] J. R. Jowsey,[3] C. C. Lee,[4] and J. W. T. Spinks[5]

Abstract

By feeding a hen repeated daily doses of sodium dihydrogen phosphate of equal P^{32} activity, eggs with high and nearly uniform P^{32} content were obtained for use in hatching experiments. Eleven eggs were incubated. The embryos and chicks that died during or after incubation were dissected and analyzed for total and specific activities in the various fractions. Two of the chicks, both females, are still living. They are being raised so that observations may be made on their ability to reproduce and the effect of the radioactivity, if any, on their offspring.

Introduction

A study by Plimmer and Scott (8) of the transformation of the phosphorus compounds in the egg to the phosphorus compounds in the chick showed that vitellin disappeared at the end of incubation. There was also a pronounced shift from a large amount of ether-soluble phosphorus and a trace of inorganic phosphorus in the egg to a relatively small amount of ether-soluble phosphorus and a large amount of inorganic phosphorus in the chick. Such results indicate that hydrolysis of organic phosphorus compounds to the inorganic form must take place during incubation.

The metabolism of lipoid phosphorus in the chick embryo has been studied by Kugler (5). The large store of phosphatides in the yolk, even shortly before the egg is hatched, as observed by Kugler (5), may lead one to suppose that the embryo might utilize these phosphatides directly in the building up of organs that contain such substances. The validity of this supposition has been tested by Hevesy *et al.* (4) by incubating eggs, each of which had received an injection into the white of P^{32}-labeled inorganic phosphate. It was found that at various stages of incubation, the phosphatide phosphorus extracted from the embryo always showed high specific activity while that extracted from the yolk was hardly active at all. The phosphatide molecules present in the embryo could not, therefore, have been taken from the yolk only, but must have been synthesized in the embryo.

The distribution of P^{32} in incubated eggs has also been studied by Dixon (2). This author administered the activity by injection of labeled potassium dihydrogen phosphate into the yolks of fertile eggs that had been incubated for five days. From the sixth day of incubation to the day of hatching, the P^{32} in the embryo was found to increase from 3% to 90% of the dose injected. The high activity observed in the allantoic and amniotic fluids showed that

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within 24 hr. following injection a shift of P^{32} from the yolk to the allantois and amnion takes place, and, as the embryo grows, the rate of uptake of phosphate from the allantois and amnion is much greater than from the yolk.

The injection of labeled phosphate into the egg, as employed by Dixon (2) and by Hevesy *et al.* (4), affords a convenient means for studying the role played by the phosphorus of inorganic phosphate in the metabolism of the chick embryo. It is well known, however, that P^{32} may be incorporated into the phosphorus compounds of the egg by administration of labeled phosphate to the hen (1, 7). The hatchability of such eggs has, as yet, not been studied. Recently, Spinks *et al.* (10) showed that after they fed a hen repeated daily doses containing an equal amount of P^{32}, for two weeks, the activity in the eggs became practically constant. This provides an excellent method for obtaining eggs of high and nearly constant activity for hatching experiments. This communication reports the results of such experiments with eggs from a hen that had received 40 daily doses of equal P^{32} activity.

Experimental

A Barred Plymouth Rock hen was isolated in a separate pen for the experiment. For a period of 40 days, the daily breeding mash was mixed with a solution of sodium dihydrogen phosphate containing 9450 registers per minute* (2.12 mc.) of P^{32} activity and fed to the hen in the morning. The decay of the P^{32} was taken into account by increasing the volume of the active solution so that the same amount of activity was fed every day. In the afternoons, when all the feed had been consumed, a Barred Plymouth Rock male was placed in the pen. Three males were used alternately throughout the 40 day period so as to ensure fertility of the eggs.

The eight eggs laid in the first two weeks following the first feeding were analyzed for total activity in the white + yolk. This involved the wet ashing of an aliquot of the white + yolk with nitric and perchloric acid, precipitation of the phosphorus as the phosphomolybdate, conversion to magnesium ammonium phosphate, and final ignition to magnesium pyrophosphate before the activity was counted. The detailed procedure has been reported previously (10). The total phosphorus was also determined, using a small aliquot of each ash solution, according to the colorimetric method of Shelton and Harper (9).

Eleven more eggs, all of which were fertile, were laid between the 15th and 46th day after the first feeding. These were incubated under standard conditions. The chicks that died during or after incubation were dissected into various fractions, as shown in the accompanying tables; these were wet ashed and the total activity and total phosphorus were determined for each fraction.

** One register per minute on the mechanical counter is equal to 128 radioactive disintegrations counted. The actual number of beta disintegrations is equal to the number of counts multiplied by a factor of 3.9 (counter efficiency was measured using an RaD–RaE standard from the National Bureau of Standards, Washington).*

When necessary, 5 to 10 mgm. of inactive ammonium phosphate were added to the aliquot being used for the determination of radioactivity to act as carrier before the phosphorus was precipitated. From the two chicks that are normal and still living, droppings were collected, twice a week, wet ashed, and the radioactivity and total phosphorus found. The analysis of the droppings was discontinued when the activity had decayed to a small value.

Results

The results of the analyses of the white + yolk of the first eight eggs as well as the total recovery of P^{32} from all fractions of the embryos or chicks are given in Table I. All activities tabulated in this and other tables in this

TABLE I

TOTAL RECOVERY OF P^{32} FROM UNINCUBATED AND INCUBATED EGGS

Column I is the time of oviposition in hours after the first feeding of P^{32}.
Column II is the total activity as of the day of oviposition in reg. per min.
Column III is the total recovery expressed as % of the daily P^{32} dosage.
Column IV is the total phosphorus in mgm.

Egg No.	I	II	III	IV	Remarks
1	9	2	0.02	73.4	White + yolk analyzed
2	45	31	0.33	83.0	"
3	70	149	1.58	79.6	"
4	172	756	8.00	76.4	"
5	195	819	8.66	77.8	"
6	222	837	8.85	80.1	"
7	269	980	10.35	81.0	"
8	313	966	10.22	87.4	"
9	413	971.6	10.27	71.28	Embryo died on about the 18th day of incubation
10	528	1050.3	11.10	68.89	Embryo died on about the 18th day of incubation
11	556	964.6	10.20	73.89	Chick killed on the day of hatching
12	606	1048.4	11.08	73.58	Chick died on the day of hatching
13	646	1048.7	11.08	67.35	Chick died two days after hatching
14	701	Lost on wet ashing			Chick died on the day of hatching
15	886	1017.8	10.77	83.60	Embryo died on about the 7th day of incubation
16	912				Chick living
17	982				Embryo died on about the 18th day of incubation—used for preliminary trials in radioautography
18	1009	1004.2	10.63	83.32	Chick died on the day of hatching
19	1108				Chick living

paper are based on the day of oviposition, correction factors having been applied to allow for the decay of the activity from the day of oviposition to the day of analysis. The total recoveries shown in Table I are also expressed as % of the daily dosage of P^{32} fed and are plotted in Fig. 1. From Fig. 1,

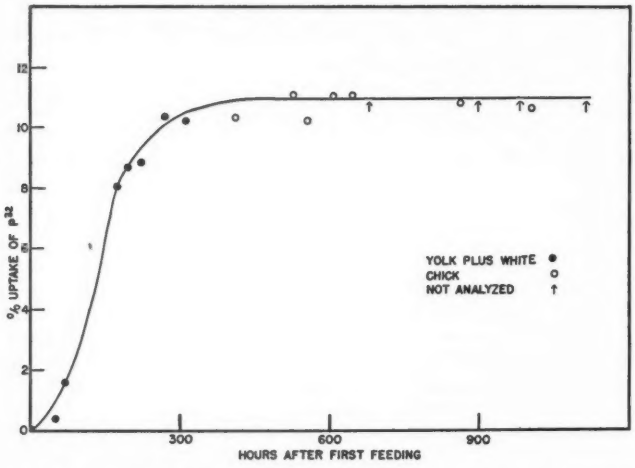

FIG. 1. *Recovery of P^{32} in successive eggs (or chicks) expressed as a percentage of the P^{32} fed on any one day. Same activity of P^{32} fed as sodium dihydrogen phosphate for 40 successive days. (Chicks not analyzed indicated by an arrow.)*

it may be seen that the total activity in each of the incubated eggs on the day of oviposition was in the order of 11% of the daily P^{32} dosage.

All of the 11 eggs (No. 9 to 19) incubated were fertile. The first chick that hatched was from egg No. 11. It showed no apparent abnormalities. It was killed and dissected on the day of hatching. Two other chicks, hatched from eggs Nos. 16 and 19, were normal and healthy; these are still living. Results of the analyses of their droppings are tabulated in Table II and plotted in Fig. 2.

The embryo from egg No. 15 died on about the seventh day of incubation. Results obtained from this egg are given in Table III. After incubation for about 18 days, the embryos from eggs Nos. 9, 10, and 17 died. Of these, the embryo from egg No. 17 was used for preliminary trials in radioautography. Those from eggs Nos. 9 and 10 were analyzed and the results are tabulated in Table IV. The ages of these four embryos were approximated by comparing the state of their development with diagrams given in Ewing's *Handbook of Poultry Nutrition* (3).

The chicks from eggs Nos. 12, 14, and 18 died on the day of hatching. Some of the fractions of the chick from egg No. 14 were lost during wet ashing.

TABLE II

ACTIVITY OF DROPPINGS OF CHICKS HATCHED FROM EGGS NOS. 16 AND 19—CHICK FROM EGG NO. 16 WAS HATCHED ON SEPT. 4, 1948; CHICK FROM EGG NO. 19 WAS HATCHED ON SEPT. 14, 1948

Column I is the period during which the sample was collected, expressed in days after hatching.
Column II is the P^{32} activity as of the day of oviposition in reg. per min.
Column III is the total phosphorus in mgm.
Column IV is the specific activity as of the day of oviposition in reg. per min. per mgm. P.

Chick from egg No. 16				Chick from egg No. 19			
I	II	III	IV	I	II	III	IV
0– 7	71.2	50.8	1.40	0– 8	74.5	41.6	1.79
8–11	96.4	47.2	2.04	9–12	50.8	71.5	0.71
12–15	88.0	62.8	1.40	13–15	22.4	66.2	0.34
16–18	49.0	57.2	0.86	16–19	36.0	63.2	0.57
19–22	66.5	70.3	0.95	20–22	22.6	69.4	0.33
23–25	42.2	68.8	0.64	23–26	32.6	70.3	0.46
26–29	39.6	73.8	0.54	27–29	32.8	66.1	0.50
30–32	26.2	67.5	0.39	30–33	19.3	71.5	0.27
33–36	34.7	71.5	0.49	34–36	12.3	64.2	0.19
37–39	28.2	68.2	0.41				
40–43	29.4	70.3	0.42				
44–46	21.7	70.5	0.31				

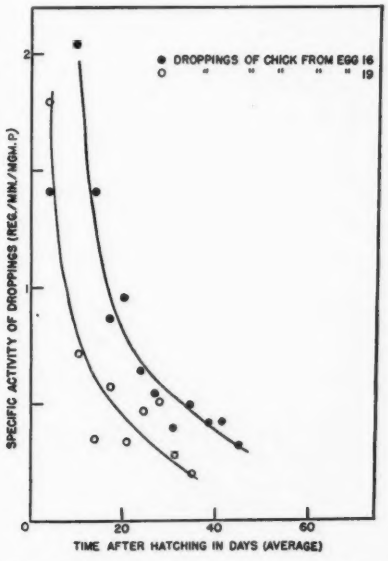

FIG. 2. *Specific activity of droppings from radioactive chicks plotted against time after hatching.*

TABLE III

ACTIVITY OF EMBRYO FROM EGG NO. 15—EMBRYO DIED ON ABOUT THE SEVENTH DAY OF INCUBATION

Column I is the activity of the corresponding fraction as of the day of oviposition in reg. per min.
Column II is the activity expressed as % of the total recovered activity.
Column III is the total phosphorus in mgm.
Column IV is the specific activity of the corresponding fraction as of the day of oviposition in reg. per min. per mgm. P.
Column V is the ratio of the specific activity of the fraction to the overall specific activity.

Fraction	I	II	III	IV	V
Viscera	3.5	0.34	0.11	31.80	2.62
Carcass	8.2	0.80	0.55	14.90	1.22
Embryonic fluids and membranes	14.1	1.38	1.04	13.55	1.11
Yolk and white	992	97.48	81.90	12.11	0.996
Total	1017.8	100.00	83.60		
Overall specific activity	1017.8/83.60 = 12.16 reg. per min. per mgm. P				

TABLE IV*

ACTIVITY OF EMBRYOS FROM EGGS NOS. 9 AND 10—EMBRYOS DIED ON ABOUT THE 18TH DAY OF INCUBATION

Fraction	Embryo from egg No. 9					Embryo from egg No. 10				
	I	II	III	IV	V	I	II	III	IV	V
Liver	11.6	1.19	1.17	9.92	0.73	8.2	0.78	0.86	9.54	0.63
Brain	13.4	1.38	1.28	10.40	0.76	13.3	1.27	1.71	7.78	0.51
Heart	3.1	0.32	0.34	9.12	0.67	1.5	0.14	0.21	7.15	0.47
Gizzard muscles	7.3	0.75	0.92	7.94	0.58	6.6	0.63	0.91	7.25	0.48
Skin and feathers	31.7	3.26	2.56	12.38	0.91	46.8	4.46	3.06	15.30	1.00
Leg muscles	19.3	1.99	2.05	9.42	0.69	23.9	2.27	2.11	11.33	0.74
Tibiae	35.3	3.64	3.81	9.27	0.68	49.1	4.68	5.10	9.63	0.63
Remainder of viscera	15.9	1.64	1.65	9.65	0.71	24.9	2.37	2.23	11.16	0.73
Remainder of carcass	356	36.63	26.38	13.50	0.99	437	41.60	26.00	16.80	1.10
Yolk sac	358	36.84	27.70	12.92	0.95	439	41.80	26.70	16.43	1.08
Embryonic fluids and membranes	120	12.35	3.42	35.10	2.57					
Total	971.6	100.00	71.28			1050.3	100.00	68.89		
Overall specific activity	971.6/71.28 = 13.63 reg. per min. per mgm. P					1050.3/68.89 = 15.27 reg. per min. per mgm. P				

* *For explanation of column numbers, see Table III.*

Consequently, the analysis of this chick was not completed. The results of the analyses of the chicks from eggs Nos. 12 and 18, together with those of the chick from egg No. 11, are tabulated in Table V.

TABLE V*

ACTIVITY OF CHICKS FROM EGGS NOS. 11, 12, AND 18—CHICK FROM EGG NO. 11 WAS KILLED ON THE DAY OF HATCHING, CHICKS FROM EGGS NOS. 12 AND 18 DIED ON THE DAY OF HATCHING

Fraction	Chick from egg No. 11					Chick from egg No. 12					Chick from egg No. 18				
	I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	V
Blood	11.7	1.21	1.31	8.93	0.69	20.0	1.91	1.58	12.66	0.89	—	—	—	—	—
Liver	16.8	1.74	1.91	8.80	0.68	27.0	2.57	1.61	16.77	1.18	12.4	1.24	1.47	8.44	0.70
Brain	18.7	1.94	1.94	9.65	0.74	22.4	2.14	1.75	12.80	0.90	17.7	1.76	2.04	8.68	0.72
Heart	3.5	0.36	0.56	6.25	0.48	4.9	0.47	0.36	13.60	0.96	4.3	0.43	0.46	9.35	0.78
Gizzard muscles	17.0	1.76	1.75	9.73	0.75	21.7	2.07	1.91	11.36	0.80	15.4	1.53	1.76	8.76	0.73
Skin and feathers	23.4	2.42	1.91	12.25	0.94	31.6	3.01	2.68	11.80	0.83	31.3	3.12	2.97	10.53	0.87
Leg muscles	39.0	4.04	4.53	8.61	0.66	44.2	4.22	2.68	16.50	1.16	40.2	4.00	3.13	12.84	1.06
Tibiae	54.3	5.63	6.10	8.90	0.68	74.0	7.05	5.63	10.50	0.74	64.1	6.39	7.25	8.58	0.73
Remainder of viscera	30.2	3.13	3.00	10.06	0.77	31.1	2.96	2.58	12.05	0.85	16.8	1.67	1.88	8.95	0.74
Remainder of carcass	472.5	49.0	32.0	14.75	1.13	521	49.7	34.25	15.22	1.08	518	51.56	34.3	15.10	1.25
Yolk sac	277.5	28.77	18.88	14.70	1.13	250	23.9	18.55	13.50	0.95	284	28.30	28.06	10.12	0.84
Total	964.6	100.00	73.89			1048.4	100.00	73.58			1004.2	100.00	83.32		
Overall specific activity	964.6/73.89 = 13.04 reg. per min. per mgm. P					1048.4/73.58 = 14.24 reg. per min. per mgm. P					1004.2/83.32 = 12.06 reg. per min. per mgm. P				

* *For explanation of column numbers, see Table III.*

The chick from egg No. 13 died two days after hatching. Results of its analysis are given in Table VI.

TABLE VI*

ACTIVITY OF CHICK FROM EGG NO. 13—CHICK DIED TWO DAYS AFTER HATCHING

Fraction	I	II	III	IV	V
Liver	22.3	2.13	2.03	10.98	0.71
Brain	20.4	1.94	1.80	11.32	0.73
Heart	7.7	0.73	0.44	17.49	1.12
Gizzard muscles	20.2	1.93	2.45	8.25	0.53
Skin and feathers	42.3	4.03	3.36	12.60	0.81
Leg muscles	43.7	4.17	3.31	13.20	0.85
Tibiae	59.8	5.70	3.78	15.80	1.01
Remainder of viscera	36.4	3.47	2.43	14.97	0.96
Remainder of carcass	583	55.60	29.50	19.75	1.27
Yolk sac	196	18.69	16.80	11.67	0.75
Droppings	16.9	1.61	1.45	11.65	0.75
Total	1048.7	100.00	67.35		
Overall specific activity	1048.7/67.35 = 15.57 reg. per min. per mgm. P				

* *For explanation of column numbers, see Table III.*

Discussion

Of the 11 fertile eggs incubated, one embryo died on the seventh day of incubation, three embryos died on the 18th day of incubation, three chicks died on the day of hatching, one chick died two days after hatching, one apparently normal chick was sacrificed on the day of hatching, and two apparently normal chicks are still living. The mortality, although very high, could still be considered within the limit observed for ordinary eggs since in the average mortality curve a maximum is reached on about the 18th day of incubation. The fact that two of the chicks could be kept alive indicates that, under the conditions of the experiments reported in this paper, the radioactivity has no apparent effect on the hatching of the egg and the normal growth of the chick. These two chicks are females. They are being raised so that observations may be made on their ability to reproduce and the effect of the radioactivity, if any, on their offspring. Inspection of the amount of P^{32} excreted by these two chicks (Table II) indicates that the chick from egg No. 19 probably contained much less radioactivity. This is not surprising since egg No. 19 was laid six days after the termination of the daily feeding of P^{32}.

From the embryos and chicks that were analyzed, radioactivity was found in all fractions examined. As is to be expected, the greatest amount of activity was found in the tissues that contain the largest quantity of phosphorus. Thus the fraction denoted "remainder of carcass", which consisted

of all the bones except the tibiae and all the flesh except the leg muscles, contained about 40% of the total activity recovered from the embryos that died on the 18th day of incubation, about 50% of the total activity recovered from the chicks that died on the day of hatching, and about 55% of the total activity recovered from the chick that died two days after hatching. The P^{32} in the yolk sac decreased from approximately 40% of the total activity in the embryos that died on the 18th day of incubation to less than 20% in the two-day-old chick. This is compatible with the well known fact that the yolk sac is rapidly absorbed during the first few days of life of the chick.

The specific activity (registers per min. per mgm. P) and the ratio of the specific activity of the fraction to the overall specific activity (the overall specific activity is the sum of activities divided by the sum of total phosphorus for all fractions of a given chick) as well as the distribution of the activity as a % of the total activity recovered are tabulated in Tables III, IV, V, and VI. A low value for the ratio of the specific activity of a given fraction to the overall specific activity is indicative of a dilution of the radiophosphorus. It is interesting to note that of all the fractions of the embryo from egg No. 9, which died on the 18th day of incubation, the embryonic fluids and membranes fraction has the highest specific activity. In the dissection of the embryo from egg No. 10, which also died on the 18th day of incubation, the yolk sac was accidentally broken and its content mixed with the embryonic fluids. However, the combined yolk sac plus embryonic fluids and membranes fraction also showed a relatively high specific activity. In a study of the relative abundance of different acid soluble phosphorus compounds in the embryonic fluid, Kugler (6) concluded that there is a high inorganic phosphorus content in the allantoic fluid. This finding, coupled with the observed high specific activity of the embryonic fluids and membranes fraction and the high rate of uptake of phosphate from the allantois and amnion found by Dixon (2), suggest that phosphorus compounds of high specific activity present in the egg, such as vitellin (1), may be hydrolyzed to inorganic phosphate, transported to the allantois and thence absorbed by the embryo. Such a mechanism would be in accord with the conclusion of Hevesy *et al.* (4) that practically all phosphorus atoms present in the various compounds of the embryo must pass through the stage of inorganic phosphorus.

With the limited number of chicks analyzed, no final interpretation can be made of the P^{32} distribution and the specific activities in the liver, brain, heart, etc. More experiments will be carried out on the distribution of P^{32} in chicks of different ages and in embryos at various stages of development.

Acknowledgment

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A KEY TO THE EMBRYOLOGICAL DEVELOPMENT OF *MELANOPLUS BIVITTATUS* (SAY), *M. MEXICANUS MEXICANUS* (SAUSS.), AND *M. PACKARDII* SCUDDER[1]

By R. W. Salt[2]

Abstract

The embryological development of three species of grasshoppers is described in gross aspect by means of a key that relates morphological development to the duration of incubation at 25° C. The three species are so similar that they are treated as one. The rate of development and stage of entering diapause are compared with those of *M. differentialis*.

A comprehensive illustrated account of the embryological development of *Melanoplus differentialis* (Thos.) at 25° C. was published by Slifer in 1932 (2). In 1936 Steele (4) published a briefer account of development in embryos of *Austroicetes cruciata* Sauss. Possibly as a result of some similarity between these two species and the popularity of *M. differentialis* eggs as laboratory test animals, it is often assumed that their eggs are representative of acridid eggs generally. Moore (1), however, points out that both *M. bivittatus* and *M. mexicanus mexicanus* differ from *M. differentialis* in respect to embryonic development and diapause. In the present study three species of *Melanoplus* were found to be so similar to each other in embryological development as to be virtually the same, yet greatly different from that reported for *M. differentialis* and *A. cruciata*. The purpose of this paper, however, is not so much to point out differences among species as to establish a convenient means of reference to embryonic development similar to that used by Slifer (2), for use in further studies.

Materials and Methods

Newly laid eggs of *Melanoplus bivittatus*, *M. m. mexicanus*, and *M. packardii* were obtained from caged adults and incubated on moist plaster of Paris at 25° C. The younger embryos were dissected from the eggs for observation but the older ones were observed *in situ* and *in vivo*, using the excellent method of chorion removal described by Slifer (3).

Results

The accompanying key makes use of morphological characters by which the course of development can be transposed into time of incubation at 25° C. From the fourth day of incubation onwards these characters are readily observable under a binocular dissecting microscope. During the first three days the embryos are too small for this method, and therefore the first three days are omitted.

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Although the three species exhibited minor differences of size and appearance there was no observable difference in development or its rate. For the purpose at hand, therefore, they may be treated as one.

KEY TO THE DEVELOPMENT OF EMBRYOS OF *Melanoplus bivittatus*, *M. m. mexicanus*, AND *M. packardii* AT 25° C.

"Tail" about same length as diameter of head	4 days
"Tail" about twice as long as head; no other differentiation into body regions	5 days
"Tail" about three times as long as head; body vaguely differentiated into four regions	6 days
Differentiation into protocephalic, protognathal, thoracic, and abdominal regions distinct; no detail in head	7 days
Antennae, labrum, mouth parts, and legs appear as distinct knobs; no abdominal segmentation	8 days
Segmentation appearing in mouth parts and legs; abdomen distinctly segmented, with appendages appearing as vague knobs; labrum not notched	9 days
Labrum notched; abdominal appendages appear as distinct knobs, with first pair (pleuropodia) larger but not greatly dissimilar to the others	10 days
Pleuropodia trilobed; other abdominal appendages still knoblike; no antennal segmentation	11 days
Hind legs beginning to fold into N-shape	12 days
Hind legs folded into N-shape; distal half of antennae shallowly segmented	13 days
Posterior rim of eyes faintly pink; embryo half the length of egg but narrow; thorax one-third width of egg. Anatrepsis now completed	14 days
Revolution of the embryo in progress; red in eyes forms thin crescents	15 days
Revolution completed; head pointing anteriorly; embryo half the length of egg, thorax three-quarters of egg width (16- to 20-day stages identifiable through the wet chorion by location, size, and coloration of the eyespots)	16 days
Embryo two-thirds of egg length; eyespots more distinct	17 days
Embryo three-quarters of egg length; yolk engulfment advanced	18 days
Yolk engulfment and dorsal closure complete; embryo full length; eyes red on upper margins only; hind femora reaching Abd. IV or V	19 days
Eyes red on half of surface; hind femora reach Abd. VI or VII	20 days
Eyes dark red on all but lower rim; mandibular teeth, lacinial teeth, and tarsal claws unpigmented and difficult to see	21 days
(DIAPAUSE OCCURS HERE IF AT ALL)	
Mandibular teeth pigmented enough to be distinctly seen; eye pigmentation complete	22 days
Mandibular teeth dark piceous; lacinial teeth, tarsal claws, and rows of spines on hind tibiae lightly pigmented	23 days
General pigmentation darker; all spurs, spines, and teeth dark piceous to black; morphological detail complete; apparently ready to hatch	24 days

The relationship between development and time as given in the key should not be interpreted as rigid or precise, but rather as an optimum. Though most embryos developed on schedule up to the onset of diapause, many fell behind for unknown reasons. At least some of this retardation is thought to be caused by handling and other artificialities of laboratory rearing. The postdiapause period is described as three days. Actually the measurement of this period is made uncertain by the difficulty of determining exactly when diapause is ended, or more specifically, when postdiapause development begins. From observation of developing embryos in which diapause was of

very short duration, a postdiapause period of about three days, during which time pigmentation steadily increased, seemed to be about optimal. However, hatching does not necessarily take place as soon as the embryo reaches the 24-day stage. It appears in most cases that a stimulus to hatch may be necessary; if so, its nature is not known.

Discussion

In comparison with *Melanoplus differentialis* (2), two differences are outstanding. The rate of development of *M. differentialis* at 25° C. is much slower and diapause occurs at a much earlier stage. In 21 days *M. differentialis* has completed anatrepsis and enters diapause. Embryos of *M. bivittatus*, *M. m. mexicanus*, and *M. packardii* complete anatrepsis in 14 days, or 1.5 times as fast as *differentialis*. They continue development and enter diapause on the 21st day at a developmental stage far beyond that of *differentialis*.

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WATER UPTAKE IN EGGS OF *MELANOPLUS BIVITTATUS* (SAY)[1]

By R. W. Salt[2]

Abstract

Eggs of *Melanoplus bivittatus* (Say) possess a special water-absorbing area, the hydropyle, similar in function to that of *M. differentialis* (Thos.). The extent of hydration was measured as eggs of *M. bivittatus* developed at 25° C. on contact moisture. The total amount of water absorbed amounts to about 60% of the original weight of the egg. Absorption is negligible for the first four days, is maximal on the eighth and ninth days, and later may exhibit a second period of minor increase and decline, which varies in time of occurrence. About 88% of the total absorption occurs during anatrepsis. Eggs denied contact moisture from the time of oviposition develop to the end of anatrepsis and remain viable for long periods, provided desiccation is not severe. When contact moisture is provided, such eggs hydrate very rapidly and continue development with a retardation of two or three days. Extra moisture from the environment is essential for the revolution and katatrepsis of the embryo.

Introduction

Eggs of *M. bivittatus* absorb water from the environment. The site and extent of absorption, and its influence on embryological development, constitute the studies reported here.

The Site of Absorption

The presence of a hydropyle such as described by Slifer (6) was verified for *M. bivittatus*. Following the method found most satisfactory by her, the posteriors of 20 eggs were coated with "liquid solder". Twenty eggs with the anterior ends coated and 10 untreated eggs served as controls. All of the eggs were from the same pod, which had been kept at 25° C. and 90% R.H. for 20 days following oviposition. They were thus denied contact moisture and had ceased development at the end of anatrepsis.

After two days on moist filter paper the untreated eggs and those with the anterior ends sealed were noticeably swelled; by the fourth day all of them appeared fully hydrated and on the fifth day the eye spots showed each egg to contain a 16- or 17-day embryo. As the solder held its shape, the eggs treated at the anterior end swelled posteriorly until it seemed they would burst. They continued healthy for some time and either hatched or entered diapause. Meanwhile the eggs sealed at the posterior or micropyle end showed no change in size or shape for six days. The solder was then softened with ethyl acetate and scraped off, after which the chorions were removed in sodium hypochlorite (7). When these 20 eggs were exposed to contact moisture (vertical, hydropyle-down, to ensure contact in the absence of the moisture-conducting chorion) nine promptly hydrated and developed. Failure of the remainder to develop was attributed to injury in removing the solder.

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Although transpiration through the general cuticular surface is not disproved, it would appear to be very limited if not absent. The main site of absorption is the micropylar area, as already shown in *M. differentialis* (6) and *Rhodnius prolixus* Stal. (1).

The Course of Absorption During Embryological Development

Isolated newly laid eggs were incubated on moist filter paper at 25° C. Each egg was weighed within 24 hr. of oviposition and every second day thereafter for 20 days on a torsion spring balance (150/0.1 mgm). On the 16th, 18th, and 20th days the age of each egg was determined by observing the position of the eye spots. Because weight increase was to be related to development, only those eggs that developed on schedule (4) are included in the analysis. On the basis of Bodine's observations on *M. differentialis* (3) it is assumed that weight increase equals water uptake.

The average weights of each group of eggs are listed in Table I and the accumulation percentage increases in Table II. As the variance among eggs of the same pod was not significant, only the means are listed in Table I.

TABLE I

MEAN WEIGHTS IN MGM. OF EGGS DEVELOPING ON SCHEDULE AT 25° C. ON CONTACT MOISTURE

Series	No. of eggs	Days										
		0	2	4	6	8	10	12	14	16	18	20
A	12	4.12	4.14	4.18	4.38	5.30	6.18	6.37	6.52	6.55	6.67	6.78
B	13	4.11	4.14	4.18	4.42	5.15	5.93	6.30	6.35	6.49	6.55	6.59
C	9	3.77	3.77	3.77	3.97	4.80	5.74	5.88	5.94	6.06	6.17	6.17
D	10	4.12	4.12	4.14	4.16	4.94	5.86	6.21	6.32	6.41	6.47	6.47
E	7	4.34	4.34	4.34	4.57	5.39	6.29	6.57	6.71	6.81	6.93	6.94
F	8	4.61	4.62	4.69	4.85	5.81	6.50	6.59	6.64	6.85	6.96	6.98
G	22	4.86	4.86	4.86	5.30	6.20	7.10	7.37	7.47	7.70	7.76	7.81
H	47	3.84	3.84	3.85	3.94	4.68	5.43	5.77	6.00	6.10	6.17	6.23
Mean		4.16	4.17	4.19	4.38	5.18	5.99	6.30	6.44	6.57	6.65	6.69

The same data are presented graphically in Fig. 1, in which it should be noted that the time axis is simultaneously embryonic development and days of exposure to contact moisture, because all eggs developed on schedule. Water uptake is negligible for about four days, after which it increases rapidly to a maximum on the eighth and ninth days, followed by a rapid decline. In seven of the eight groups a second, minor increase in absorption rate also appears. This is not uniform in point of time, however, with the result that combining the data of all groups (Fig. 1, I) obscures the second mode. The second rate increase does not appear to be related directly to embryonic age although it may be related indirectly as a characteristic of the micropylar absorption area.

TABLE II

MEAN ACCUMULATIVE PERCENTAGE WEIGHT INCREASES OF EGGS DEVELOPING ON SCHEDULE AT 25° C. ON CONTACT MOISTURE

Series	No. of eggs	Days									
		2	4	6	8	10	12	14	16	18	20
A	12	0.5	1.5	6.3	28.7	50.0	54.7	58.3	59.0	61.9	64.6
B	13	0.7	1.7	7.5	25.3	44.3	53.3	54.5	57.9	59.4	60.3
C	9	0.0	0.0	5.3	27.3	52.2	56.0	57.6	60.8	63.6	63.6
D	10	0.0	0.5	1.0	19.9	42.2	50.8	53.6	55.6	57.1	57.1
E	7	0.0	0.0	5.3	24.2	44.9	51.4	54.6	56.9	59.7	59.9
F	8	0.2	1.7	5.2	26.0	41.0	43.0	44.0	48.6	51.0	51.4
G	22	0.0	0.0	9.1	27.6	46.1	51.7	53.7	58.5	59.7	60.7
H	47	0.0	0.3	2.6	21.9	41.4	50.2	56.2	58.9	60.7	62.2
Mean		0.2	0.7	5.3	24.5	45.2	51.5	54.8	58.0	59.9	60.8

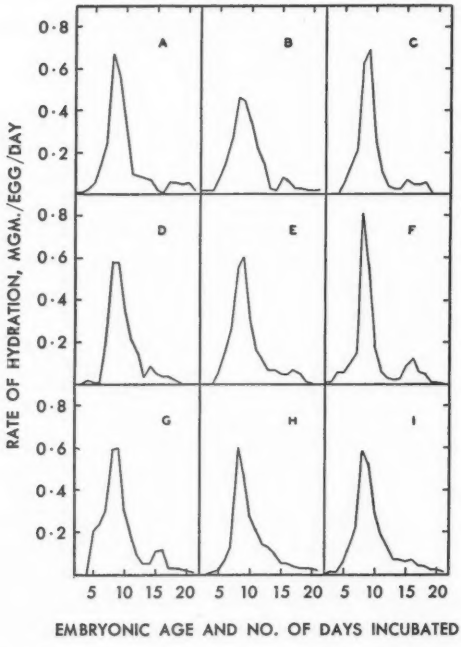

FIG. 1. *Rates of hydration in milligrams per egg per day of eggs developing on schedule at 25° C. on contact moisture. A to H—means of samples from single pods; I—mean of all eggs.*

The proportional increase in weight of the eggs bulked in one group may be followed in Fig. 2. The period of greatest absorption lies between the 6th and 12th days, during the latter part of anatrepsis, but without any apparent

relationship to embryonic growth. The dotted line represents the theoretical levelling-off of weight had the second absorption period been absent; the actual increase is thereby accentuated.

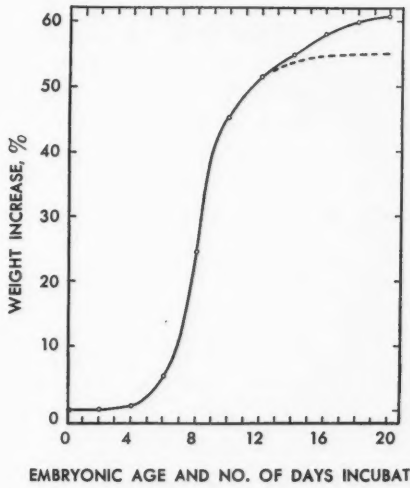

FIG. 2. *Mean percentage weight increase of 128 eggs developing on schedule at 25° C. on contact moisture. Solid line—observed data; dotted line—theoretical position if second absorption period were not present.*

The experimental conditions described above allowed continuous, maximum absorption. In order to determine how necessary this is to the egg and to the embryo, experiments limiting the water supply were carried on. It was first established that eggs exposed to 25° C. and 50% R.H. from the time of oviposition survived for at least 20 days, during which time they completed anatrepsis but never developed further. In order to avoid both excessive dehydration and contact moisture, large numbers of eggs were placed at 25° C. and 90% R.H. Over 90% of these eggs completed anatrepsis and remained viable for at least 60 days, some living as long as 200 days. Here again, as at 50% R.H., development ceased at the end of anatrepsis. When contact moisture was supplied to such eggs they absorbed it very quickly. Table III shows that 92 such eggs absorbed nearly 50% of their weight in two days and in six days had gained as much as normally-incubated eggs. The hydration is so fast that the bimodal characteristic noted in Fig. 1 is not discernible.

Age records were kept for the eggs represented in Table III, which were handled as individuals, as well as for several hundred others that were not weighed. Most of the embryos reached the 16-day stage on the fourth day of hydration and practically all of the remainder by the fifth day. Thus the average retardation is somewhat over two days.

TABLE III

WEIGHT INCREASE AND DEVELOPMENT OF 92 EGGS ON CONTACT MOISTURE AT 25° C. AFTER 30 DAYS AT 25° C. AND 90% R.H. FROM DATE OF OVIPOSITION

	Days hydrated					
	0	1	2	3	4	6
Average weight, mgm.	4.50	5.80	6.70	6.95	7.03	7.20
Accumulative increase, %	—	28.9	48.9	54.5	56.2	60.0
Predominant embryonic age	14	—	—	—	16	18

It might be supposed that the weight of the egg would give a rough indication of its embryonic development. This is far from being the case. Variation in weight was found to be very great for any age group, even for eggs taken from the same pod. Including eggs up to five days of age, by which time hydration is negligible, 1856 eggs from 85 pods ranged from 3.0 mgm. to 5.5 mgm., each extreme being represented in newly laid eggs. Comparable variations are found throughout the range of embryonic growth. Thus, although hydration results in large weight increases, these may be obscured by normal size variation.

Discussion

In 1929 Bodine (3) published smoothed curves showing that eggs of *M. differentialis* absorb water during their development. No details were given regarding amounts, but it was indicated that there was a fairly steady increase in weight and water content during the active developmental period. The increase was interrupted by diapause, during which time a steady level of water content was maintained. In 1938 Slifer (6) published curves of prediapause and postdiapause weight increases for the same species. These represent averages of large numbers of eggs and reveal that absorption is very slight during the first week. This is in agreement with findings for *M. bivittatus* when a correction is made for the differences in rate of development. (*M. differentialis* reaches the end of anatrepsis in 21 days at 25° C. (5); *M. bivittatus* in 14 days. Hence the latter's rate of development is about 1.5 times that of *M. differentialis*.) By the time anatrepsis is complete, *M. differentialis* has absorbed about 0.7 mgm. per egg; *M. bivittatus* 2.28 mgm. (Table I). During katatrepsis, *M. differentialis* absorbs about 3.2 mgm.; *M. bivittatus* about 0.3 mgm. The pattern of absorption is thus entirely different in the two species; *differentialis* absorbs water chiefly during katatrepsis and *bivittatus* during anatrepsis.

It is of interest to compare the relationship between hydration and diapause in the two species. Slifer (8) has shown that in *M. differentialis* diapause can be broken by a 30-min. immersion in a fat solvent such as xylol, which acts by dissolving a lipid layer at the hydropyle and allowing the entrance of water. *M. bivittatus* is similar in that it also requires water from the environment

before katatrepsis can proceed, but is different in that the absorption normally takes place during anatrepsis and has no connection whatsoever with diapause. We have seen that if young eggs of *M. bivittatus* are denied contact moisture they cease development at the end of anatrepsis, which is the stage at which *M. differentialis* enters diapause. They cannot develop further until they have absorbed water, and if this is not forthcoming they survive for rather long periods but are ready at any time to absorb water and resume development. Thus they are not in a state of true diapause although the result is similar. The condition is comparable to that of a chilled insect that is able to resume normal activity and development as soon as it is warmed.

Although Slifer warns against the assumption that hydration is the only factor involved in the resumption of growth in *M. differentialis*, it is surely a very important one. In contrast, it is difficult to see how hydration could possibly play a part in diapause in *M. bivittatus* eggs. In this species diapause starts on the 21st day at 25° C. (corresponding to about 32 days for the slower *M. differentialis*). By this time hydration is complete or practically so, and the embryo is almost completely developed. Nor does the bimodal character of the absorption curve bear any apparent relation to diapause. Though variable in its time of occurrence, the second mode occurs at least several days before the onset of diapause. Slifer's postdiapause absorption curve (6) does not give any indication of being bimodal; if actually so, it is obscured by time variation as in Fig. 1, I and Fig. 2.

Birch and Andrewartha (2) give brief records of the weight of eggs of *Austroicetes cruciata* Sauss. at certain stages of embryonic development. From these data it appears that hydration extended over the entire period and was about 40% completed by the end of anatrepsis. In the latter regard it differs from both *M. bivittatus* and *M. differentialis*. The same authors show that the eggs of *A. cruciata* absorb moisture during diapause. However, diapause in this species is much different from that in *Melanoplus*; it lasts during the entire course of anatrepsis except the first few days and may extend into katatrepsis also. Thus embryonic development progresses, though slowly, during diapause.

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